CANADIAN JOURNAL OF RESEARCH

VOLUME 18 MAY, 1940 NUMBER 5

CONTENTS

SECTION C.—BOTANICAL SCIENCES

SECTION D.—ZOOLOGICAL SCIENCES

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

Publications and Subscriptions

The Canadian Journal of Research is issued monthly in four sections, as follows:

A. Physical Sciences
B. Chemical Sciences
C. Botanical Sciences
D. Zoological Sciences

For the present, Sections A and B are issued under a single cover, as also are Sections C and D, with separate pagination of the four sections, to permit separate binding, if desired.

Subscription rates, postage paid to any part of the world (effective 1 April, 1939), are as follows:

	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
C and D	2.50	0.50
Four sections, complete	4.00	—

The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

National Research Council, Ottawa, Canada.

Notice to Contributors

Fifty reprints of each paper are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges.

Publications and Subscriptions

The Canadian Journal of Research is issued monthly in four sections, as follows:

A. Physical Sciences
B. Chemical Sciences
C. Botanical Sciences
D. Zoological Sciences

For the present, Sections A and B are issued under a single cover, as also are Sections C and D, with separate pagination of the four sections, to permit separate binding, if desired.

Subscription rates, postage paid to any part of the world (effective 1 April, 1939), are as follows:

	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
C and D	2.50	0.50
Four sections, complete	4.00	—

The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

National Research Council, Ottawa, Canada.

Notice to Contributors

Fifty reprints of each paper are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 18, SEC. C. MAY, 1940 NUMBER 5

THE EFFECT OF NUTRIENT SALTS IN ORGANIC MERCURIAL SEED DISINFECTANTS ON THE GERMINATION AND EARLY GROWTH OF WHEAT[1]

BY N. H. GRACE[2]

Abstract

Wheat seed was treated by dusting with a series of seed disinfectants containing 5% of ethyl mercuric halide (80 Br : 20 Cl) and three concentrations of each of KNO_3, KH_2PO_4, NH_4KHPO_4, and $Ca(H_2PO_4)_2 \cdot H_2O$. This method of applying nutrient salts failed to effect any general stimulation to germination or early growth. In one experiment with Marquis wheat, KNO_3 accelerated germination without increasing the final value, and all but KH_2PO_4 increased root weight three weeks after planting. In two subsequent experiments with only KNO_3 added to the mercurial, but with four varieties of wheat, conducted at different temperatures, no stimulation was observed.

Recent work on the treatment of plant cuttings and seeds with dusts containing a small amount of growth stimulating chemicals has suggested that other factors, such as nutrient salts, might be used effectively in the same manner (3, 4). Preliminary experiments on the use of nutrient salts in seed disinfectant dusts gave results that suggested slight stimulation to the germination and early growth of wheat. The present communication gives the results of a more detailed investigation in which several nutrient salts were used in an organic mercurial seed disinfectant.

Experimental

The mercurial dusts all contained 5% of ethyl mercuric halide* in a good grade of commercial talc, and were subjected to a grinding mix for about 15 hr. in a laboratory ball mill. Four nutrient salts, at three concentrations, were used with the organic mercurial. The salts were KNO_3, KH_2PO_4, $Ca(H_2PO_4)_2 \cdot H_2O$, and NH_4KHPO_4. The dusts with KH_2PO_4 contained 2, 5, and 10% of the salt. The amounts of KNO_3 added gave the same amounts of potassium as were contained in the dusts at 2, 5, and 10% of KH_2PO_4. Similarly, the amounts of the other two salts used gave the same phosphate concentration as was provided by KH_2PO_4 at its three levels of concentration.

[1] *Manuscript received January 26, 1940.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. N.R.C. No. 903.

[2] *Biochemist.*

* *The ethyl mercuric halide used in these experiments was prepared by a method developed in the Division of Chemistry, National Research Laboratories, Ottawa, by Dr. A. Cambron. This procedure yielded a product consisting of 80% ethyl mercuric bromide and 20% ethyl mercuric chloride.*

Three experiments were carried out; in the first, 227-gm. samples of Marquis wheat were treated with the dusts at the rate of one-half ounce of dust per bushel of seed. Both seed and dust were placed in a glass bottle which was subjected to the action of a mechanical roller for a period of five minutes, a procedure which ensured thorough mixing. In the second and third experiments, 113.5-gm. samples of Dawson's Golden Chaff, Red Fife, Renown, and Reward wheats were treated at the rate of one-half ounce of dust per bushel of wheat with four dusts of the series. These dusts comprised organic mercury alone and organic mercury with the three levels of potassium nitrate.

The treated seeds were planted in sterilized soil in small 4 × 4 in. cardboard flats, 50 seeds to each flat, the unit in the experiments. In the first experiment the four salts were used at three concentrations, along with a control containing organic mercury alone, of which four flats were included in each replicate in order to provide averages of equal weight for all dosages and the control. The 16 flats receiving the various treatments were completely randomized in respect of position within each block, and there were 10 replicate blocks in all, which involved a total of 8000 seeds. In the second and third experiments there were also 10 blocks, each block being square and containing the varieties in compact sub-blocks of four units. Varietal sub-blocks were randomized as to position within main blocks, and the four treatments occurred at random within each of the four sub-blocks. In all three experiments the blocks were flanked by a row of flats containing untreated seed; this procedure reduced border effects.

In Experiment 1 the seed was planted November 30, 1938, in a greenhouse room maintained at approximately 55° F., and daily germination counts were taken as soon as germination was observed, which was eight days after planting. These daily counts were continued until germination was virtually complete. The plants were washed out of the soil 21 days after planting, held in an oven at 95° C. for two hours, and conditioned for one week in the laboratory. The exhausted seeds were picked off and the air-dry root and stem weights for the plants from 50 seeds were determined.

In Experiment 2, the seed was planted January 25, 1939, in a greenhouse room which ranged in temperature between 65 and 70° F. Daily germination counts and air-dry root and stem weights, 24 days after planting, were taken in the manner already described. In Experiment 3, the seed was planted February 22, 1939, in a greenhouse room maintained between 50 and 55° F. Daily germination counts were determined and also the air-dry weight of plants (stems and roots together) 33 days after planting.

In Experiment 1, the germination data were analysed statistically over the first three daily counts, the last five daily counts, and over all eight counts. In Experiments 2 and 3, germination rates were computed from the daily germination counts by the method of Bartlett (1); in addition, the final germination count was also considered. All the data were subjected to analyses of variance.

Results

Experiment 1

Germination data over the first three daily counts, the air-dry weight of roots from 50 seeds, and the analyses of variance of the responses to treatment are given in Tables I, II, and III, respectively. Potassium nitrate increased germination over the first three daily counts, but none of the levels of KH_2PO_4, $Ca(H_2PO_4)_2$, or NH_4KHPO_4 differed significantly from the control. Stimulation from potassium nitrate treatment, 49% germination against 38.2% for the control on the third day, may be attributed to the two highest levels used, namely, 3.7 and 7.4% of salt in the mercurial dust. It is apparent from Table III that stimulation of germination, under the conditions of this experi-

TABLE I

GERMINATION OVER FIRST THREE DAYS OF MARQUIS WHEAT TREATED WITH NUTRIENT SALTS IN AN ORGANIC MERCURIAL SEED DISINFECTANT

(Experiment 1)

Nutrient salt	Transformed data[1]				Germination on the third daily count, means by salts[2], %
	Control (organic mercurial alone)	Relative concentration of nutrient salt in mercurial disinfectant		Mean for all three nutrient salt concentrations	
		1	2.5	5	
KNO_3	2.346	2.705	2.807	3.086	2.866	49.0
$Ca(H_2PO_4)_2$	2.346	2.332	2.583	2.593	2.503	38.2
KH_2PO_4	2.346	2.238	2.482	2.083	2.268	34.6
NH_4KHPO_4	2.346	2.077	2.246	1.987	2.103	30.4[2]

Necessary difference, 5% level of significance, between treatments, 0.562; treatments and control, 0.444; treatment mean and control, 0.304.

[1] *Data transformed to* $\sqrt{x + \frac{1}{4}}$ *basis (2) for analysis of variance.*

[2] *Mean germination for the control groups, with organic mercury only, on the third daily count was 38.2%.*

TABLE II

AIR-DRY WEIGHT (GRAMS) OF ROOTS FROM PLANTS GROWN IN 21 DAYS FROM 50 SEEDS, TREATED WITH NUTRIENT SALTS IN AN ORGANIC MERCURIAL SEED DISINFECTANT

(Experiment 1)

Nutrient salt	Control (organic mercurial alone)	Relative concentration of nutrient salt in mercurial disinfectant			Mean for all three nutrient salt concentrations
		1	2.5	5	
KNO_3	0.228	0.242	0.263	0.238	0.248
$Ca(H_2PO_4)_2$	0.228	0.231	0.257	0.245	0.244
KH_2PO_4	0.228	0.235	0.233	0.226	0.231
NH_4KHPO_4	0.228	0.242	0.240	0.245	0.242

Necessary difference, 5% level of significance, between treatments, 0.013; treatments and control, 0.010; treatment mean and control, 0.007.

TABLE III

ANALYSIS OF VARIANCE OF RESPONSE OF MARQUIS SEED TREATED WITH NUTRIENT SALTS IN AN ORGANIC MERCURIAL DUST DISINFECTANT

(Experiment 1)

Source of variance	D.f.	Mean square				
		Germination over			Air-dry weights ($\times 10^3$)	
		First 3 days	Last 5 days	All 8 days	Stems	Roots
Replicates	9	9.23**	37.41	236.1**	17.71**	2.03**
Treatments	12	3.11**	39.97	117.0**	3.31	1.44**
Error	138	1.19	28.83	42.5	2.24	0.60

** *Exceeds mean square error, 1% level of significance.*

ment, was confined to the first three days on which counts were made and did not extend to the subsequent five daily germination counts.

While nutrient salt treatment failed to increase significantly the weight of stems, there were some significant increases in the air-dry weight of roots. Considering the means for all three levels of the four salts, we note that all the salts but KH_2PO_4 increased root weight. All three concentrations of potassium nitrate effected an increase in root weight with the intermediate concentration having the greatest activity; the root weight fell significantly on further increase of the salt in the dust. The two highest concentrations of $Ca(H_2PO_4)_2$ gave increased root weights, but, while the weights fail to differ significantly, a falling off is suggested at the highest level. All three treatments with NH_4KHPO_4 gave increased root weights; however, the response is essentially the same at all concentrations. No significant variation in root weight was effected by KH_2PO_4 treatments.

The results suggest that the increased root weights obtained with KNO_3 may be attributed to the presence of nitrate, since KH_2PO_4 had no effect, and dusts of these two salts contained identical amounts of potassium. Similarly, the activity of the other two salts, having the same amount of phosphate as KH_2PO_4, appears to be related to the presence of calcium and ammonium nitrogen.

Since treatments with potassium nitrate gave the greatest effects on root weights, and the only stimulation to germination in the experiment, succeeding experiments were confined to this one salt. However, four varieties of wheat were employed in an effort to investigate the possibility of differential effects of treatment upon varieties.

Experiment 2

The analyses of variance for the data are given in Table IV. Highly significant varietal differences were shown for all of the properties measured. The only significant treatment effect was upon germination and this was in a negative direction. There were no interactions between varieties and treatments.

TABLE IV

ANALYSIS OF VARIANCE OF RESPONSE OF FOUR VARIETIES OF WHEAT SEED TREATED WITH POTASSIUM NITRATE IN AN ORGANIC MERCURIAL SEED DISINFECTANT

(Experiment 2)

Source of variance	D.f.	Mean square			
		Germination rate	Final germination count	Air-dry weight	
				Stems	Roots
Replicates	9	0.0259***	1.692	0.0382***	0.0132***
Varieties	3	0.4482***	43.875***	0.6885***	0.2470***
Interaction					
Varieties × replicates (error (a))	27	0.0038	3.229	0.0045	0.0015
Treatments	3	0.0144***	1.842	0.0030	0.0006
Interaction					
Varieties × treatment	9	0.0021	1.714	0.0022	0.0006
Error (b)	108	0.0021	1.713	0.0040	0.0011

*** *Exceeds mean square error, 0.1% level of significance.*

In Tables V and VI are given data for the different varieties and effects of potassium nitrate treatment on the germination rate. The effect of treatment with potassium nitrate, averaged over all four varieties, was a significant reduction in the germination rate when 7.43% of the salt was present in the organic mercurial disinfectant. Neither the 1.49% nor the 3.71% levels of potassium nitrate differed significantly from the control, though a decrease in rate was suggested by the higher of these two concentrations.

Absence of stimulation to germination, or stem and root weights, suggested that the higher temperature under which growth occurred (about 68° as compared with about 55° F. in Experiment 1) might have had an effect on

TABLE V

AVERAGE RESULTS, ALL TREATMENTS, WITH WHEAT PLANTS GROWN IN 24 DAYS FROM 50 SEEDS OF EACH OF FOUR VARIETIES

(Experiment 2)

Variety	Germination rate	Final germination count of 50 seeds planted	Air-dry weight, gm.	
			Stems	Roots
Dawson's Golden Chaff	0.828	49.9	0.95	0.41
Red Fife	0.682	48.8	0.63	0.23
Renown	0.743	47.8	0.80	0.33
Reward	0.576	47.5	0.76	0.27
Necessary difference, 5% level of significance	0.028	0.8	0.03	0.02

TABLE VI

EFFECT OF POTASSIUM NITRATE IN AN ORGANIC MERCURIAL SEED DISINFECTANT ON GERMINATION RATES OF FOUR VARIETIES OF WHEAT

(Experiment 2)

	Concentration of salt in disinfectant dust, %				Necessary difference, 5% level of significance
	0	1.49	3.71	7.43	
Rate	0.720	0.720	0.709	0.680	0.020

the results. In consequence, Experiment 3 was a repetition of Experiment 2, but with temperature held at about 55° F.

Experiment 3

Tables VII and VIII give the analyses of variance of the responses and the data for germination rates, final germination, and air-dry plant weights for four varieties of wheat. It is apparent that the only significant feature of the experiment was the difference between varieties. There were no treatment effects nor any interactions between treatments and varieties. The four varieties fell into the same order as they did in Experiment 2. In view of these results, absence of stimulation from potassium nitrate treatment in Experiment 2 cannot be attributed to the higher temperature under which growth occurred.

The addition of nutrient salts, along with an organic mercurial in a seed disinfectant dust, thus failed to effect any general stimulation to the germination and early growth of wheat. Owing to the season of the year, the two

TABLE VII

ANALYSIS OF VARIANCE OF RESPONSE OF FOUR VARIETIES OF WHEAT SEED TREATED WITH POTASSIUM NITRATE IN AN ORGANIC MERCURIAL SEED DISINFECTANT

(Experiment 3)

Source of variance	D.f.	Mean square		
		Germination rate	Final germination count	Air-dry weight of plants
Replicates	9	0.0024	6.334	0.3685***
Varieties	3	0.2061***	73.990***	3.1114***
Interaction				
Varieties × replicates (error (a))	27	0.0014	3.036	0.0494
Treatments	3	0.0007	3.556	0.0036
Interaction				
Varieties × treatment	9	0.0010	1.984	0.0288
Error (b)	108	0.0009	2.784	0.0172

*** *Exceeds mean square error, 0.1% level of significance.*

TABLE VIII

AVERAGE RESULTS, ALL TREATMENTS, WITH WHEAT PLANTS GROWN IN 33 DAYS FROM 50 SEEDS OF EACH OF FOUR VARIETIES

(Experiment 3)

Variety	Germination rate	Final germination count of 50 seeds planted	Air-dry weight of plants from 50 seeds, gm.
Dawson's Golden Chaff	0.682	49.4	2.34
Red Fife	0.584	48.0	1.68
Renown	0.622	46.6	2.00
Reward	0.511	46.5	1.88
Necessary difference, 5% level of significance	0.017	0.8	0.10

experiments with potassium nitrate were grown under a greater intensity of light than the first experiment, which showed some significant stimulation. Light effects may accordingly have a bearing on the results obtained. Thus, there is an indication that there are growth conditions under which nutrient salts may cause stimulation to the early germination and growth of wheat, although these conditions are not such as would be expected to occur in the field.

References

1. BARTLETT, M. S. J. Roy. Stat. Soc. (Suppl.) 4 : 137-183. 1937.
2. COCHRAN, W. G. Empire J. Exp. Agr. 6 : 157-175. 1938.
3. GRACE, N. H. Can. J. Research, C, 15 : 538-546. 1937.
4. GRACE, N. H. Can. J. Research, C, 17 : 321-333. 1939

ACTION OF MICRO-ORGANISMS ON FAT*

II. OBSERVATIONS OF UNINOCULATED GLOBULES OF TRIGLYCERIDES AND FATTY ACIDS AND OF BUTTERFAT IN AN AGAR MEDIUM[1]

By C. H. Castell[2] and E. H. Garrard[3]

Abstract

With the object of discovering the significance of the "opaqueness" surrounding so-called lipolytic colonies on fat-emulsion agar plates, detailed observations have been made on uninoculated globules of butterfat, pure triglycerides, fatty acids, and mixtures of fats and fatty acids. The following observations have been made:

At room temperature, sooner or later, fat crystals form in all butterfat globules. The rapidity with which crystals form and their general conformation and texture are capable of considerable variation. This depends upon the previous history of the butterfat, temperature, and the physical and chemical characteristics of the agar medium in which the fat is suspended.

Sodium chloride, among other substances, brings about rapid changes in the form of the globules and the texture of the crystals.

The crystals of pure fats suspended in agar are quite unlike the crystals in globules of butterfat. When these same fats are suspended in some form of oily matrix this difference disappears.

The chief difference between pure fats and fatty acids, when suspended in oil, is that there is a tendency for the fatty acids to crystallize at the fat-agar interface, while the fats do not. The lower, solid fatty acids often protrude out into the agar medium.

As well as copper sulphate and Nile blue sulphate, a large number of other dyes have been used to differentiate fatty acids from fats.

It appears probable that the phenomenon of opaqueness in the fat globules surrounding lipolytic colonies in fat-emulsion agar plates is owing, at least in part, to the rapid formation of fat crystals, as well as to the formation of fatty acids.

Introduction

In the use of oil-emulsion agars for the identification of lipolytic bacteria, two types of observations are made. An indicator, such as Nile blue sulphate, may be incorporated into the medium, and lipolysis indicated by changes in the colour of this dye; and lipolysis may be indicated by physical changes occurring in the individual fat globules and observed as either a clear zone or an opaque area surrounding the colony in question. In a previous paper (3) examination was made into the significance which can be attached to the colour changes of certain dyes used in this manner. This present investigation will deal with the physical changes occurring in the individual fat globules.

Choice of Fatty Materials

There are two general types of fats used in these tests, namely, simple triglycerides and natural fats, such as cottonseed oil or butterfat. Anderson (1) recommended tributyrin in nutrient agar and used this medium for examining the microbial content of milk, cream, water, and sewage. Collins

[1] *Manuscript received November 29, 1939, and as revised February 20, 1940.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario.

[2] *Research Assistant.*
[3] *Professor of Bacteriology.*

* *The first paper in this series appeared in the Iowa State Coll. J. Sci. 4 : 313-328. 1939.*

and Hammer (4) have shown that hydrolysis by bacteria of simple triglycerides was more difficult as the molecular weight increased. Long and Hammer (5) found that certain cultures commonly regarded as non-lipolytic would bring about reactions on plates containing either dispersed tripropionin or tributyrin. similar to the reaction brought about by bacteria capable of hydrolysing fats.

These observations show clearly that the action of an organism in hydrolysing or not hydrolysing one specific triglyceride does not necessarily indicate its action on others. Although the fats of lower molecular weights may give the largest number of positive tests with a group of cultures, such information might be of little value where applied to natural fats. Even with fats containing propionic and butyric acid radicals, it is unlikely that appreciable amounts will be in the form of pure triglycerides.

For these reasons it seems apparent that no one fatty material can be used universally for this purpose. As this series of papers deals as closely as possible with the action of bacteria on the fat of milk and milk products, butterfat will be the "natural" fat chiefly considered.

Microscopical examination of individual globules in the "opaque areas" surrounding a typical lipolytic colony growing in a butterfat agar emulsion medium, shows the opaqueness to be due to the formation of crystalline material. The use of polaroid discs makes this still more evident. Efforts will be made to discover the nature of these crystals. They may be fats, fatty acids, combinations of the two, or some other by-products of fat spoilage. Until this is known, the real significance of these opaque areas remains a question of doubt.

Experimental

Two series of observations have been made. The first deals with the globules formed from triglycerides and fatty acids singly and in various combinations. The second deals with globules of butterfat under various conditions. The results are then compared.

Observations on Globules of Triglycerides and Fatty Acids

These experiments were carried out with the hope of finding out whether the fats and fatty acids could be distinguished from each other under conditions comparable to the fat-emulsion-test media used for detecting lipolysis.

To 1% sterile melted agar, 3% of the fat, fatty acid, or fatty-acid–fat mixture was added. These were autoclaved at 10 lb. pressure for 10 min., and then after vigorous shaking, poured into sterile Petri dishes. They were incubated at 25° C. The following materials were used:

Triolein; tributyrin; tricaprin; tricaproin; trilaurin; trimyristin; tripalmitin; tristearin.

Triolein + trilaurin; triolein + trimyristin; triolein + tripalmitin; triolein + tristearin; triolein + tripalmitin + trilaurin; triolein + tripalmitin + trimyristin; triolein + tripalmitin + tristearin; triolein + tripalmitin + tristearin + trilaurin + trimyristin.

Oleic acid; palmitic acid; stearic acid.

Triolein + butyric acid; triolein + caproic acid; triolein + caprylic acid; triolein + lauric acid; triolein + myristic acid; triolein + palmitic acid; triolein + stearic acid; triolein + oleic acid; triolein + palmitic + lauric acids; triolein + palmitic + myristic acids; triolein + palmitic + lauric + myristic + stearic acids; triolein + palmitic acid + palmitin; triolein + lauric acid + palmitin; triolein + myristic acid + palmitin.

Added to these observations were others in which the triolein was replaced by paraffin oil, and some in which various dyes were added to the media. Details were noted and photomicrographs were made from each plate. Because of the unavoidable length of these descriptions only certain of the more important ones will be recorded in this paper.

Individual Triglycerides

There were no crystal formations in any of the globules of the four liquid fats, tributyrin, tricaproin, tricaprylin, and tricaprin. There were, however, slight differences between the globules of these fats. Those of the tributyrin sank to the bottom of the agar; those of the tricaproin and tricaprylin had a greater tendency to rise and spread out on the surface of the agar. Of the four, tricaprin had the greatest tendency to remain dispersed as small spherical globules in the agar. The globules of each of these four fats had spherical droplets within them. Many of these droplets appeared relatively unstable, disappearing when exposed to air or bright light.

Triolein differs from the fats described above in having an excessively large number of the colourless spherical droplets within the globules. These droplets in the surface globules are at first very unstable. When the cover of the Petri dish has been removed they suddenly disappear. Under the microscope this change appears to be one of dissolving rather than bursting. Several minutes after the lid of the Petri dish has been replaced, similar but very minute droplets may form again. The point of a hot needle against the edge of a surface globule causes the instant formation of countless numbers of these small spheres. When the Petri dishes have been incubated a week or more, there is a decided increase in the number and stability of these droplets. The addition of certain substances, such as Nile blue sulphate, methylene blue, or sodium chloride, reduces the number of these bodies.

Globules of the solid fats were at first spherical and homogeneous. On cooling, they crystallized and lost their spherical shape. As can be seen in the illustrations (Figs. 3 and 20), there is very little similarity between these and the crystal formations observed in normal butterfat.

Fat Mixtures

About 50% of the fats in butter are liquid at room temperature. This means that as the higher fats crystallize they do so in a matrix of oil. For this reason a series of plates was made in which the higher fats were mixed with triolein or paraffin oil. Under these conditions the fats crystallized in forms quite different from those in the agar alone. Tripalmitin and tristearin, and more especially tripalmitin with lesser amounts of tristearin, trimyristin

and trilaurin, produced crystal formations very similar to those observed in the butterfat (Figs. 21 to 23). It was always observed that crystals were never found in the agar outside of, or protruding from, the oily matrix (i.e., the globules of paraffin or triolein). In many cases the crystals were formed in the *centre* of the surface globules, rather than at the agar interface.

Although the solid fats showed considerable variation as they crystallized in the globules of triolein, there were sufficient characteristic differences to differentiate each fat microscopically. Tristearin and tripalmitin in globules of triolein frequently form crystal formations that are very similar, and in this form they are similar to the crystals that are first observed in globules of butterfat. These are shown in Fig. 24. They begin as a more or less irregularly circular mass of feathery or fern-like crystals radiating from a central point. In many ways they suggest the microscopical picture of a young actinomycete or mould colony. Later this may grow into such a compact mass that it is only with difficulty that the individual crystals can be seen. Trimyristin and trilaurin differ from tristearin and tripalmitin in having larger and coarser crystals and in forming crystal masses with much less symmetrical outline (Figs. 25 to 30).

Fatty Acids

At first, the crystals of the fatty acids forming in the globules of oil appeared similar to the corresponding fats. They are also subject to considerable variation. One noticeable difference is the tendency of the acids to crystallize at the fat agar interface (Figs. 31, 32 and 35) (i.e., on the outside of submerged globules and around the circumference of surface globules). With the softer acids the crystals frequently start at the surface and grow out into the agar (Fig. 31). This never occurs with the fats. Another difference between the crystals of fats and fatty acids is that the acids tend to form into a less compact mass. Frequently they produce separate needle-like crystals, or groups arranged in a fan-like fashion (Fig. 36). These characteristics are not invariable. Under some circumstances the acid crystals form completely within the fat, and many observations were made, especially with stearic and palmitic acids, where the crystals were formed into compact little masses, not unlike one of the forms assumed by tristearin. However, in any one plate there was always sufficient variation in crystal formation to enable even an inexperienced observer to differentiate between fats and fatty acids, and in most cases, to recognize the individual fats or fatty acids.

It was frequently observed that as the molecular weight of the acids decreased in these fat-acid mixtures, there was a greater tendency for the globules to unite and rise to the surface. Even on the surface itself, with lauric acid, there were large areas where the globules were grouped together leaving other areas free from globules altogether. This was much less noticeable with stearic or palmitic acids. But when lauric acid was added to either palmitin or palmitic acids (in triolein), this apparent increase in surface tension was evident.

THE ACTION OF COPPER SULPHATE AND OTHER INDICATORS IN DIFFERENTIATING GLOBULES OF FATS AND FATTY ACIDS AND THE REACTION OF THESE REAGENTS TO GLOBULES OF BUTTERFAT

Copper sulphate (6) Nile blue sulphate (2) methylene blue (3) and other indicators have been used in oil emulsion agars to indicate the production of fatty acids. Experiments were conducted with these indicators on uninoculated globules of triglycerides, fatty acids, and butterfat, under conditions similar to those in the oil-emulsion agar tests. The object was to test the ability of these reagents to indicate the presence of individual fatty acids, and then to compare this with their reaction on the globules of butterfat.

Plates were prepared and poured in the usual manner. Nile blue sulphate and methylene blue were added as the medium was being prepared. The saturated copper sulphate solution was flooded over the surface of the agar after the plates had been poured and hardened. The materials tested were: Stearic, palmitic, myristic, lauric, caproic, caprylic, and oleic acids and their corresponding triglycerides.

Copper sulphate: None of the fats showed any change. All the fatty acids ultimately turned blue. The liquid acids became blue on contact with the salt solution; myristic and lauric acids were doubtfully blue after 20 min. at room temperature, and definitely blue at the end of an hour; the stearic and palmitic acids were colourless at 20 min. but coloured at the end of an hour.

Nile blue sulphate: The liquid fats were coloured various shades of pink, rose, and red; the solid fats were less deeply stained and the colours were changed to shades of brownish red with tinges of purple as the melting point of the fat increased. The liquid fatty acids stained deep blue or shades of purplish blue; the solid fats stained less deeply, but still had sufficient blue colouring to enable them to be easily differentiated from the corresponding fats. These observations agree with those of Collins and Hammer (4), who have had extensive experience with this dye.

Methylene blue: None of the fats were stained; the liquid acids were blue; lauric acid was pale blue; myristic, palmitic, and stearic acids showed very little colouring.

When the temperature was raised above their melting points all the fatty acids produced instantaneous and intense colour reactions with each of these reagents.

Other indicators: Similar tests were made on caproic and caprylic acids and their corresponding triglycerides, with aqueous solutions of the following dyes: malachite green, methyl violet, basic fuchsin, neutral red, rose bengal, china blue, toluidine blue, gentian violet, saffranine O, rose-aniline hydrochloride, eosin, brilliant green, phenol-indo 2 : 6-dichlorophenol, and *p*-aminodimethylaniline monohydrochloride. In each instance the fats remained uncoloured and the acids were deeply stained. When myristic and stearic acids were added to a 1 to 1000 aqueous solution of these dyes and the temperature was raised above the melting point of the acids, they became coloured. (It is

of interest to note that all these dyes that stain the fatty acids appear to be oxidation-reduction indicators. The addition of a weak solution of sodium hydrosulphite or other reducing agent renders them colourless, and the addition of hydrogen peroxide restores the colour.)

Observations on changes occurring in uninoculated globules of butterfat

Unless otherwise stated, the butterfat used throughout these experiments was made from fresh, pasteurized, sweet cream; it was unsalted and the curd was removed by shaking the butter in warm water and separating the fat in a separatory funnel. The basic agar medium was prepared by adding 1% standardized granular Bacto agar to 1 litre of distilled water. The amount of fat used was 3% by volume. Unless otherwise stated, the medium was autoclaved at 15 lb. pressure for 15 min. Immediately before pouring the plates, flasks were vigorously shaken in order to disperse the fat in the form of small globules throughout the medium.

Observations, under varying conditions, demonstrate the fact that there are three or four main characteristic physical changes which may occur in uninoculated globules of butterfat. As these form a basis for further descriptions and may be significant when related to enzyme activity, it is necessary to give a brief description of them at the beginning.

A. Crystal Masses

When the plates are first poured, the globules of butterfat appear under the microscope as homogeneous, liquid, or semi-liquid bodies (Fig. 1). As the fat cools, crystals appear in the globules (Fig. 2). These crystals are subject to great variation, both in the form they assume and the rate at which they form. If the plates are incubated at 25° C., at the end of two or three days almost every globule will contain one or more large masses of feathery crystals which radiate from a central point (Fig. 3). These usually form first at the surface of the globule, appearing mainly in one plane; later they grow down into the fat, forming dense feathery crystal masses. After the formation of these primary crystal masses, either within them or forming a border around them, crystalline material of a different appearance may be formed. It appears as a softer wax-like material, with little or no indication of the feather-like crystal formation. That the melting temperature is one of the chief differences between these types of crystals, can be demonstrated by observing their action through a series of temperature changes (Figs. 15 and 16). The waxy-looking material melts at a lower temperature. Both types rotate polarized light. Given sufficient time, some form of the primary crystal masses invariably forms in globules of butterfat. The second type is observed less frequently and only after a longer incubation period. One characteristic was constant to all these crystal formations in the globules of butterfat. They never protruded out of the fat into the surrounding medium.

In all probability, the crystal masses described above are similar to those that Ritter (6) described as "hedgehog-like crystals" in his observations on cooked butter.

B. Submerged and Surface Globules

Apart from the temperature at which the plates are poured, and the vigour with which the medium is shaken, there are other factors that greatly influence the dispersion of the fat in the agar. The globules may remain small and spherical, with those near the surface completely or almost completely submerged in the medium; or they may have a tendency to unite, to rise to the surface, and to spread out on the top of the agar (Figs. 18 and 19). This may be owing to the formation of thin protecting films of phospholipoids or protein material around the individual globules. When the butterfat is fresh there is a greater tendency for it to remain dispersed as fine globules. The addition of even very small percentages of sodium chloride brings the globules to the surface.

This difference (whether the globules are dispersed in the medium or mainly at the surface) becomes more significant when bacterial action is considered. There are some species that appear to be more active in attacking fat when it is exposed to the air.

C. Droplets

Within the fat globules there are frequently found large numbers of small colourless spherical bodies (Figs. 9 and 12). They are associated with the liquid portions of the fat and are very abundant in globules of pure triolein. At times it is difficult to decide whether these are liquid or gaseous in nature or whether they are always the same substance. Occasionally they have been stained blue with both Nile blue sulphate and methylene blue. They vary a great deal in size, and under some conditions appear to be very unstable. Similar looking droplets appear in globules pierced by a portion of mould mycelium. In this latter instance they are apparently water droplets resulting from respiration.

Closer observation shows another type of droplet frequently found in butterfat. In fresh butterfat these droplets are darker in colour than the fat in which they are suspended. They are much smaller and more regular in size than the colourless droplets mentioned above. In size they are similar to the smaller fat globules of normal milk. Large globules may contain as many as a hundred of these bodies; smaller ones may contain none, one, or more. Careful examination shows that similar bodies are occasionally found in the agar away from the fat globules, but never in agar not containing the butterfat. At reduced temperatures these bodies crystallize and lose their spherical shape. All attempts to photograph these smaller types of droplets met with failure.

Effect of Temperature on Globules of Butterfat

Many observations were made on globules of butterfat at temperatures ranging from 5° C. (41° F.) to 37° C. (98° F.). These may be summarized as follows: Other things being equal, decrease in temperature brings about faster, finer, and more complete crystallization, with less tendency towards differentiation of crystal types. At any one temperature there appears to be

a maximum amount of crystalline material formed, but the speed at which it forms varies with the past history of the butter sample as well as with the physical and chemical characteristics of the medium in which the fat is dispersed.

At a temperature of 25° C. (77° F.) approximately 30 to 50% of the globule ultimately becomes crystalline and the crystals are relatively large and coarse. At 5° C. (41° F.) the whole globule appears to become a solid mass of crystalline material. As the crystals are finer at this temperature, there is much less distortion of the shape of the globules than when they are allowed to crystallize at higher temperatures. When samples were suddenly cooled, and after crystallization incubated for long periods at room temperature, they never became as coarse or irregular as similar samples incubated at room temperature without the pre-cooling (Fig. 17).

Addition of Various Substances to the Agar Medium

In order to encourage bacterial growth and to adjust the pH, etc., a considerable number of different materials may be added to the various fat-emulsion agars used for detecting bacterial lipase. Little consideration has been given to the effect of these added substances on the mechanism of the tests for fat spoilage, or the changes they bring about in the fat itself. It will be shown in experiments to be reported later that very small amounts of sodium chloride enable an organism, otherwise considered non-lipolytic, to bring about changes similar to a lipase-positive organism.

In this section a few observations will be made on the action of some common substances on globules of butterfat.

Sodium Chloride—Sodium chloride, even in relatively small amounts (0.5% and less) has a marked effect on the butterfat in an agar emulsion. The globules are changed in such a way that instead of remaining submerged or partly submerged in the agar, they tend to rise to the surface and spread out. The crystal masses formed in these surface globules are relatively large and very coarse (Figs. 3 and 5); differentiation of crystal types is marked; and the production of the larger colourless droplets associated with the more liquid portion of normal butterfat globules is either greatly reduced or entirely absent. On further incubation the globules tend to become very irregular (Fig. 6).

Incubation of the plates in the dark at room temperature for several weeks brings out still further differences between salted and unsalted plates. The surface globules in the medium to which the salt has been added tend to contract and leave empty a portion of the mould which they originally made in the agar (Fig. 4). In many cases the structural make-up of the globules appears to assume a definite plan. The crystal masses form dense areas around the outer surface and bottom of the globules, giving them the appearance of many little craters (Fig. 7). The central area, which at first appears empty, contains the liquid and semi-liquid portions of the globule. And this in turn, by the addition of dyes, can be shown to contain a more or less spherical, colourless, central body (Fig. 8).

As most of this crater-like formation of the globules when salt is added occurs several weeks after the plates have been poured, it has little interest as far as it pertains to the immediate use of such plates for bacteriological tests. However, it is of interest in showing the peculiar action of salt on the fat globules, and it is of further interest when it is realized that the enzymes of certain types of micro-organisms bring about similar changes in the fat after a few days' incubation.

Compared to the unsalted butter, the addition of salt hastens the bleaching of the globules and the production of tallowy odour from the plates. So far, it has been difficult to determine whether this accelerated oxidation is owing only to the fact that on the salted plates the globules are more exposed to the atmosphere, or whether some other factor is involved.

Other Substances—The addition of 5% normal sodium hydroxide, sodium bisulphite, peptone, and many other substances, has an effect similar to sodium chloride in bringing about some change (probably in surface tension) which caused the globules to rise to the surface of the medium and spread out. Further changes varied considerably with the amount and the nature of the substance added.

Variations in pH of the medium, brought about by the addition of either sodium hydroxide or lactic acid, caused changes in size and texture of the crystals and in the form of the globules (Figs. 10, 11, and 12). The variations are greater as the value increases or decreases from somewhere near neutrality. At the lower pH range, the crystal masses are large and coarse; slightly above neutrality, from pH 7.2 to 8.5, they are less and finer.

Many other experiments were tried, such as thorough and repeated washing of the butter, prolonged shaking as an emulsion in a vibration machine (the results of which are shown in Fig. 14), heat treatments, and the effect of age of the butter (Fig. 13). All brought about changes in the globules that were fairly constant.

Discussion

The observations described have indicated that the crystals formed from the solid triglycerides in a matrix of oil are very similar to those found in globules of butterfat. They are quite different when they crystallize in agar alone. Although the crystalline structures of these fats are capable of considerable variation, there are characteristic differences by which the higher fats, tristearin and tripalmitin, can be readily differentiated from the softer fats, trimyristin and trilaurin, when observed in these microscopic oil globules. In a similar way the corresponding fatty acids can be distinguished.

It is not always possible to differentiate between the crystals of fats and fatty acids as they form in these globules. There are, however, some characteristic differences that frequently enable almost instant differentiation. These characteristics become more pronounced as the number of carbon atoms in the acids decrease. The fat crystals tend to form wholly within the oil globule. The fatty acids tend to crystallize around the surface of the oil

globules, and with the softer fats they grow out into the agar. Such orientation is in agreement with our knowledge of the affinity between water and one end of the fatty acid molecule; this relation does not exist with the triglycerides.

Another frequent difference is that the fats always form crystal masses in which the crystals radiate out from a central point. Fatty acid crystals frequently form singly or in fan-shaped masses, or in other less compact forms.

Copper sulphate, Nile blue sulphate, methylene blue, and many other indicators gave colour reactions with fatty acids, which do not occur with either the pure triglycerides or globules prepared from butterfat.

Globules of butterfat dispersed in an agar medium, which are at first homogeneous, have been observed to go through a series of easily observed changes. These consist mainly of the formation of various types of crystal masses within the globules, the appearance of minute spherical bodies in the more liquid portion of the fat, and finally what appear to be changes in surface tension, determining whether the globules remain small and spherical, dispersed throughout the agar, or partially unite to form large flat globules on the surface of the medium.

Sudden chilling of the globules at low temperature brings about the complete crystallization of the fatty material; the crystals are very fine and the globules do not lose their spherical shape. When crystallization is slower the crystals are larger and coarser, and the globules lose their spherical shape owing to distortion by these crystals. Slow crystallization brings about differentiation of crystalline material into at least two types. The last formed is the first to melt as the temperature rises.

Globules of fresh unheated butterfat in pure agar to which no other material has been added remain "normal" for the greatest length of time. The addition of many other substances, washing, heating, severe shaking, and ageing of the butter, all tend to hasten crystallization of the fat and bring about other changes in the globules.

By comparing the crystals that form in globules of butterfat with those of the pure triglycerides formed under similar conditions, there is every indication that the crystals in the butterfat are those of the higher melting-point fats. The amount of these crystals forming in the butterfat agrees approximately with the known percentage of the higher fats in butter. It is also apparent that the "opaqueness" in fat globules following bacterial activity may be owing to the rapid crystallization of fats as well as to the formation of fatty acids. Observations have been made on differences in the formation of fat and fatty acid crystals. These, together with the colour reactions of the fatty acids in the presence of copper sulphate, or any one of a large number of oxidation-reduction indicators, will enable the differentiation of fat crystals from those of fatty acids in globules that have been subjected to bacterial activity.

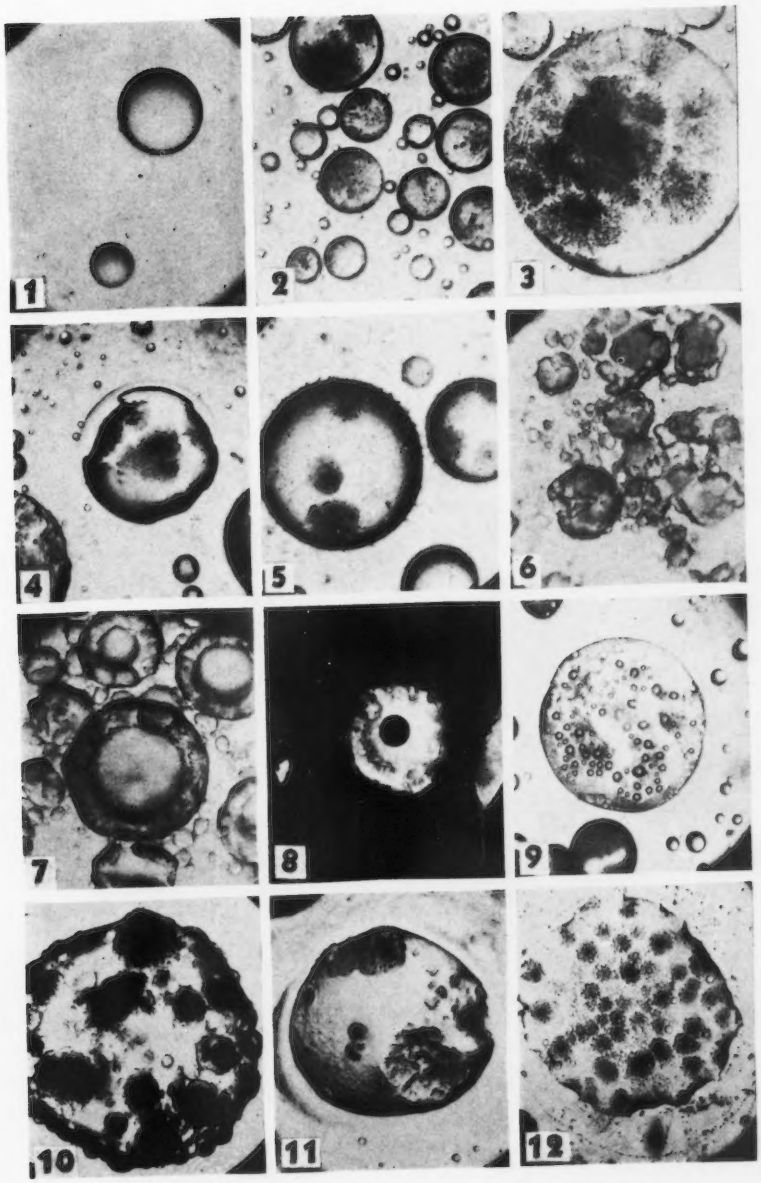
Apart from their application to further studies on the action of micro-organisms on fats, some of the observations recorded here appear to have an application in other fields. The texture of butter and other fatty foods is determined to a considerable extent by crystal structure. Fats in globular form afford a splendid opportunity for observing and photographing crystals, and changes occurring in crystals. They can be handled with facility and easily subjected to variation in environment. An example of the practical use of such observations is seen in the rapid pre-cooling of the butterfat globules incubated at room temperature. They are more uniform and contain finer crystals than those incubated at the same temperature without the short pre-cooling. This may have its counterpart in the texture of lard or butter.

References

1. ANDERSON, J. A. J. Bact. 27 : 69. 1934.
2. BERRY, J. A. J. Bact. 25 : 433. 1933.
3. CASTELL, C. H. and BRYANT, L. R. Iowa State Coll. J. Sci. 4 : 313-328. 1939.
4. COLLINS, M. A. and HAMMER, B. W. J. Bact. 27 : 473-485. 1934.
5. LONG, H. F. and HAMMER, B. W. Iowa State Coll. J. Sci. 12 : 385-395. 1938.
6. RITTER, W. Schweiz. Milchztg. 64 : 429-430. 1938.
7. TURNER, R. H. J. Infectious Diseases, 44 : 126. 1929.

PLATE I, FIGS. 1 – 12. *Globules of butterfat. All figures ×80.* FIG. 1. *Submerged globules before the formation of any crystalline material.* FIG. 2. *Typical globules of normal butterfat after 2 or 3 days' incubation at 25° C.* FIG. 3. *Crystal formation in large surface globule. A very small amount of sodium chloride had been added to the agar in which this globule was suspended, to enlarge the size of the globules and hasten crystallization.* FIG. 4. *Addition of 5% sodium chloride. After 3 days' incubation at 25° C. Note coarse crystal masses and shrinkage of the globule in the agar mould.* FIGS. 5 AND 6. *Addition of 5% sodium chloride. After 20 hours' and 8 days' incubation respectively. Note appearance of disintegration.* FIG. 7. *Globules 28 days old dispersed in beef extract agar. These typical "crater" forms are either on or near the surface. They frequently form in a much shorter period.* FIG. 8. *Similar to Figs. 5 and 6, except that india ink has been flooded over the plate. This demonstrates the spherical bodies within the oily centre of these crater forms. Frequently the india ink very quickly adheres to the surface of these spherical bodies.* FIG. 9. *Droplets in a surface globule of partially oxidized butter. Note similar droplets in Fig. 12.* FIG. 10. *pH about 4.4. Agar not jellied. Globules float at surface.* FIG. 11. *pH about 6.0.* FIG. 12. *pH about 8.8. A surface globule. Note shrinkage of the globule, finer crystal masses and droplets.*

PLATE I



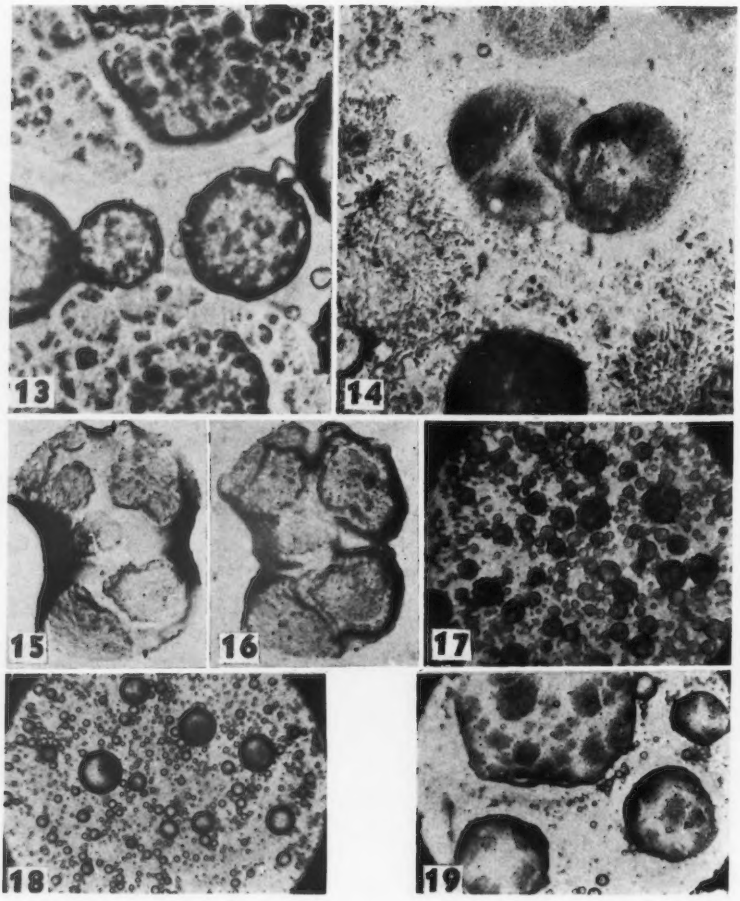


PLATE II, FIGS 13 – 19. FIG. 13. *Globues made from old rancid butter, at the end of 24 hr. Crystal formation was very rapid.* ×*160.* FIG. 14. *Butterfat added to 20% brine solution and shaken vigorously for 20 min. Fat washed free of salt and dispersed in agar. Greatly enlarged, showing the variation of crystal bodies in a portion of a surface globule.* ×*440.* FIGS. 15 AND 16. *The same globule of normal butterfat at different temperatures. Decrease in temperature from 25° C. to below 15° C. caused the formation of the wax-like edges around the crystal masses.* ×*160.* FIG. 17. *This plate was placed in the ice box for 1 hr. immediately after being poured, and then incubated for 8 days at 25° C. The initial rapid crystallization resulted in finer crystals and more uniform globules.* ×*32.* FIG. 18. *Normal butterfat globules at 24 hr.* ×*32.* FIG. 19. *The same to which 5% peptone had been added.* ×*32.*

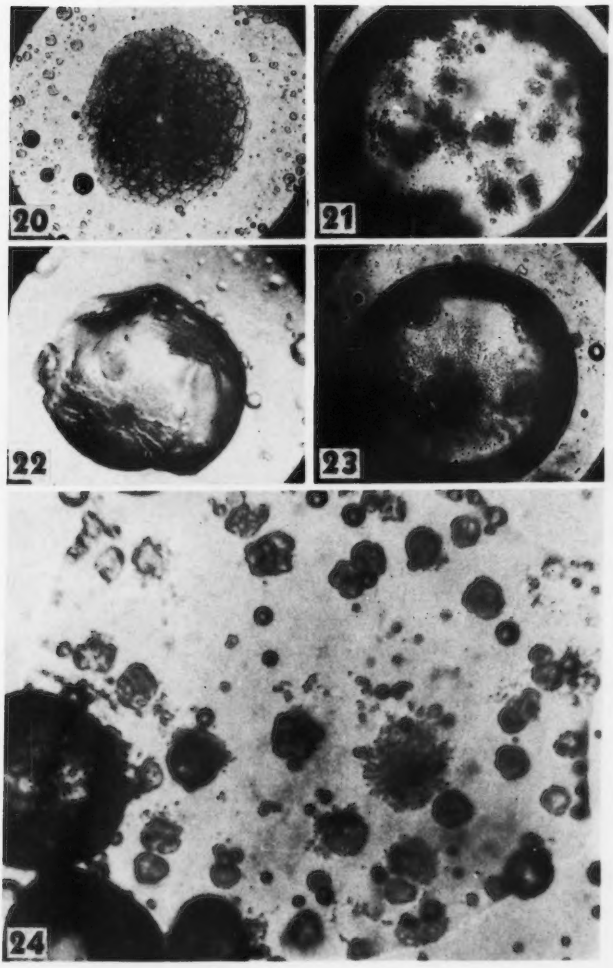


PLATE III, FIGS 20 – 24. FIG. 20. *Tripalmitin alone in agar.* ×*80.* FIG. 21. *A large surface globule of a mixture of tristearin and triolein.* ×*160.* FIG. 22. *Surface and submerged globules of a mixture of tripalmitin and triolein.* ×*160.* FIG. 23. *Globules of mixtures of tristearin, tripalmitin, and triolein.* ×*160.* FIG. 24. *Surface and submerged globules of a mixture of tristearin and triolein. In the large surface globule there are both the commonly illustrated compact crystal masses and the feathery type more frequently met with in butterfat globules.* ×*440.*

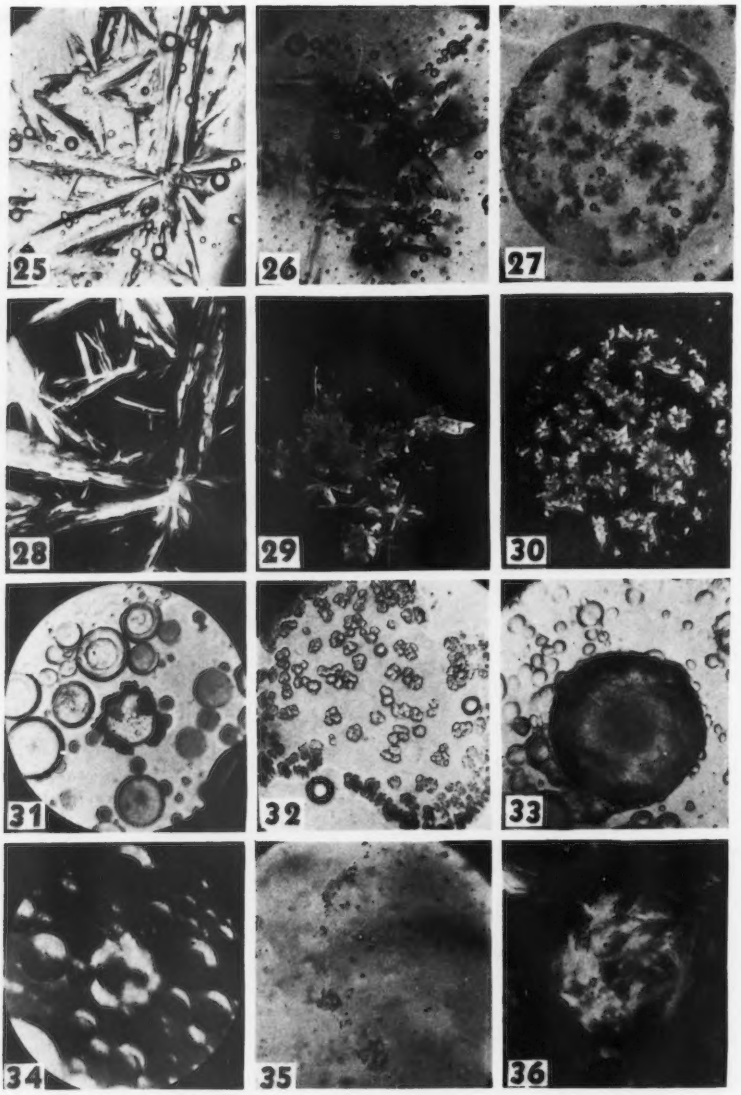


PLATE IV, FIGS. 25 – 36. *Crystals of pure fats and fatty acids in globules of triolein photographed with and without polaroid discs.* FIGS. 25 AND 28. *Trilaurin and triolein.* ×*160.* FIGS. 26 AND 29. *Trilaurin and triolein.* ×*80.* FIGS. 27 AND 30. *Trimyristin and triolein.* ×*80.* FIGS. 31 AND 34. *Myristic acid and triolein.* ×*80.* FIG. 32. *Stearic acid and triolein.* ×*160.* FIGS. 33 AND 36. *Stearic, palmitic, myristic, and lauric acids in globules of triolein.* ×*80.* FIG. 35. *Large surface globules of myristic acid and triolein.* ×*80.*

VARIETAL DIFFERENCES IN BARLEYS AND MALTS

IX. CARBOHYDRATE FRACTIONS OF BARLEY AND THEIR CORRELATIONS WITH TOTAL NITROGEN AND 1000-KERNEL WEIGHT[1]

By C. A. Ayre[2], H. R. Sallans[3], and J. A. Anderson[4]

Abstract

Samples of 12 varieties of barley from 12 experimental stations in Canada were analysed for starch, barley extract, and crude cellulose plus lignin. Nine six-rowed varieties averaged 3.4% lower in starch, 3.7% lower in extract, and 1.4% higher in cellulose plus lignin than three two-rowed varieties. Maximum and minimum values of the means for the six-rowed varieties were: starch, 55.0% and 52.4%; extract, 77.2% and 74.3%; and crude cellulose plus lignin, 11.3% and 10.1%. Environment had a significant effect. Maximum and minimum values for station means were: starch, 59.2% and 51.7%; extract, 80.8% and 73.6%; crude cellulose plus lignin, 11.0% and 9.6%.

Correlation coefficients of carbohydrate fractions with total nitrogen were not significant between varieties but highly significant within varieties for starch, $r = -0.95$, and extract, $r = -0.91$. Partial coefficients, independent of nitrogen between starch and 1000-kernel weight, were significant: between varieties, 0.62; within varieties, 0.75. Those with crude cellulose plus lignin did not attain significance.

Starch and barley extract are closely associated within varieties, $r = 0.961$, and between varieties, $r = 0.982$. The insoluble cellulose-lignin fraction gave negative inter-varietal associations with starch, $r = -0.952$, and barley extract, $r = -0.968$. Similar relations within varieties are obscured in the simple coefficients but the corresponding partials independent of total nitrogen are significant.

Introduction

As progress was made in the investigation of barley and malt properties and of the relations among them, it appeared that the possibilities for predicting malt extract from the results of barley analyses might repay further study. It therefore seemed desirable to make provision for a more adequate investigation of this subject by adding the results of determinations of various carbohydrate fractions to the data already collected on other properties of Canadian barleys.

Since starch makes the greatest contribution to the extract yield of malt, a determination of it was essential. Of the various methods available, that of Lintner-Schwartz (6), which involves dispersion in sulphuric acid and subsequent polarization, was selected as the most suitable for the present study. It is doubtful whether the method determines starch with accuracy, but it is

[1] *Manuscript received January 18, 1940.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Published as Paper No. 175 of the Associate Committee on Grain Research of the National Research Council of Canada and the Dominion Department of Agriculture. and as N.R.C. No. 904.

[2] *Formerly Biochemist, National Research Laboratories, Ottawa; now Assistant Chemist, Food and Drugs Laboratory, Department of Pensions and National Health, Toronto.*

[3] *Biochemist.*

[4] *Formerly Biochemist, National Research Laboratories, Ottawa; now Chief Chemist, Grain Research Laboratory, Board of Grain Commissioners, Winnipeg.*

comparatively rapid, yields reproducible results and thus has several advantages for large scale routine investigations. In the present instance it is more important to know whether the Lintner-Schwartz method yields results that are valuable for prediction purposes than to know if it determines starch alone and is not affected by other minor constituents of the barley.

It also seemed advisable to determine total barley extract. Most previous investigators have attempted to devise methods that will give identical values for barley extract and the extract of the malt made from it. Owing to the loss of potentially extractable barley matter during malting, this does not seem to be logical since, to attain these conditions, barley extract must be under-estimated. If a constant relation can be shown to exist between barley and malt extracts, it is of no consequence whether the values are identical since prediction can be made by calculation. In these circumstances a method that would determine all the potentially extractable material of the barley was sought for the present investigation.

To realize these conditions it was essential to grind the barley finely, and a ball mill was used for this purpose. After some experimental work, a modification of the Lüers-Miller (5) method was applied to the ground barley. It involves mashing with the addition of preparations of liquefying and saccharifying enzymes and a subsequent determination of the specific gravity of the resulting wort. To obtain additional information, the total nitrogen of the wort was determined. It was thus possible to calculate the non-protein extract yield of the barley and this could be compared with starch content.

Since Bishop's work (3, 4) has shown that malt extract is inversely correlated with the insoluble carbohydrates of the barley, it also seemed desirable to determine this fraction. Bishop's method, treatment with boiling 0.5 *N* sulphuric acid followed by boiling 0.5 *N* sodium hydroxide, determines an anomalous fraction and for this reason is not particularly attractive. A more specific fraction can be obtained by the method of Waksman-Stevens (7), which determines roughly the combined cellulose plus lignin. This method is no less empirical, but it appeared that it might yield additional information that could not be obtained merely by repeating Bishop's work.

In the present study, 144 samples of barley were analysed for starch, extract, extract nitrogen, and the acid resistant carbohydrate residue which can be called crude cellulose plus lignin. A statistical study of the relations among these constituents and total nitrogen and 1000-kernel weight was also undertaken. The data and statistics are more or less self-explanatory and discussion of them has therefore been reduced to a minimum.

Materials and Methods

Materials

The barley samples used in the present investigation were described in detail in Part I of this series of papers (1). Briefly, they consisted of 144 samples representing 12 varieties grown at each of 12 widely separated experimental stations in Canada. The varieties are listed in Table I and the stations in Table II. For the purpose of the determinations described in the

present paper, the samples were ground first in a Wiley mill and subsequently for 24 hr. in a ball mill. To provide a check on the precision of analyses, duplicate determinations were made on one-third of the samples. These were selected at random after imposing the limitation that four samples of each variety and four samples from each station should be chosen.

Starch

A 1.25-gm. sample of barley flour was weighed into a 100-ml. glass centrifuge tube. It was then worked into a smooth paste with 2 ml. of 95% alcohol, and 25 ml. of sulphuric acid (sp. gr. 1.4) was added with vigorous stirring to prevent the formation of lumps. The tube was placed in a water bath at 20° C. and the mixture stirred at intervals of five minutes. At the end of one hour, 20 ml. of sulphuric acid (sp. gr. 1.3) and 5 ml. of a 2% solution of phosphotungstic acid were added with vigorous stirring. The tube was then centrifuged and the slightly cloudy supernatant liquid was decanted through a filter paper (Whatman No. 44). Six polarimetric readings were made on the clear filtrate. The average reading was converted to per cent starch by multiplying by the factor 10.1, and the resulting figure corrected for the moisture content of the original sample. The standard error of a single determination was 0.25% starch, or 0.5% of the mean value, 54.3%.

Barley Extract

A 15-gm. sample of barley contained in a brass mashing beaker was made into a smooth paste with 50 ml. of distilled water, and 25 ml. of an 0.8% solution of "rapidase"* was added. The beaker was held at 20° C. overnight in a controlled water bath. In the morning, the thin mash was boiled on a hot plate for 10 min., with constant stirring. After cooling in a mash bath to 45° C., 25 ml. of a 1.2% solution of malt diastase and sufficient distilled water to cover the stirrer were added. The temperature was then raised to 48–50° C., held there for 10 min., and then increased at a rate of one degree per minute to 75° C., where it was held for 30 min. The mixture was cooled to room temperature, made up to a weight of 135 gm., stirred and transferred to a dry centrifuge bottle. After centrifuging, the supernatant liquid was decanted through a folded filter paper and the specific gravity of the filtrate was determined. Blank determinations were made on the enzyme solutions and, after correcting for this blank, barley extract was calculated in the usual manner. The standard error of a single determination proved to be 0.23% in barley extract, or 0.3% of the mean value, 76.0%.

The nitrogen in 25 ml. of the barley extract was determined by the Kjeldahl method and is reported as a percentage of the original sample of barley.

Crude Cellulose Plus Lignin

The small amount of material remaining in the mash beaker was washed into the centrifuge bottle, which already contained the bulk of the residue from the barley extract determination. The material was mixed with a

* *The rapidase and malt diastase preparations used in this investigation were supplied through the courtesy of the Wallerstein Laboratories, 180 Madison Ave., New York City, U.S.A.*

small quantity of water, centrifuged, and the supernatant washings decanted. The residue was mixed with 100 ml. of a 2% solution of hydrochloric acid and boiled under a reflux condenser for five hours. For this purpose, the centrifuge bottle was immersed in an oil bath maintained at 117° C. The mixture was then cooled and centrifuged. After decanting the clear supernatant liquid, the solid residue was transferred to a Buchner funnel containing a tared filter paper (Whatman No. 41) and washed six successive times with boiling water. The acid-insoluble material and filter paper were dried for five hours in an air oven at 100° C. and then weighed. The nitrogen content of the residue was determined by the Kjeldahl method, and non-protein acid-resistant residue was calculated after converting nitrogen to protein with the factor 6.0. The non-protein acid-resistant fraction is reported as crude cellulose plus lignin. The standard error of a single observation was 0.13%, or 1.3% of the mean value, 10.4%.

Results and Discussion

Varietal Differences

The results of the investigation are summarized in Table I as mean values for each variety over all 12 stations. Owing to the differential effect of environment on varieties, these did not fall in the same order at each station. Analyses of variance were necessary to determine whether the differences between the means for individual varieties could be considered significant. The results of these analyses are given in Table III but are summarized in the last line of Table I as necessary differences between means required for a 5% level of significance, i.e., for odds of 19 to 1 that real differences exist between varieties.

TABLE I

MEAN VALUES FOR EACH VARIETY, FOR STARCH, CRUDE CELLULOSE-LIGNIN, AND BARLEY EXTRACT

Class	Variety	Starch, %	Crude cellulose-lignin, %	Extract, %	Extract nitrogen, %
Six-rowed rough-awned	O.A.C. 21	53.6	10.5	75.3	0.64
	Mensury, Ott. 60	53.5	10.3	75.2	0.65
	Olli	54.7	10.1	77.2	0.71
	Peatland	54.3	10.2	75.9	0.69
	Pontiac	53.0	11.1	74.6	0.63
Six-rowed smooth-awned	Nobarb	55.0	10.2	76.5	0.56
	Regal	52.7	10.9	74.3	0.62
	Velvet	52.4	11.0	74.5	0.62
	Wisconsin 38	53.2	11.3	74.4	0.58
Two-rowed rough-awned	Charlottetown 80	56.9	9.4	78.5	0.66
	Hannchen	57.3	9.2	79.5	0.67
	Victory	56.8	8.9	79.0	0.64
Mean, over all varieties		54.5	10.3	76.2	0.64
Maximum spread		4.9	2.4	5.2	0.15
Necessary difference, 5% level		0.66	0.32	0.64	0.013

A comparison of the actual differences between varietal means, with the necessary differences required for a 5% level of significance, shows clearly that there are real differences between varieties for all properties measured. This was to be expected since varietal differences have been shown for all properties reported in the present series of papers.

The data show clear-cut differences between two-rowed and six-rowed varieties. While the former are higher in starch and barley extract, they are lower in crude cellulose plus lignin. This is in agreement with the findings of Bishop (3), who attributes the higher yield of malt extract obtained from two-rowed varieties to this fact. Among the six-rowed varieties there appears to be some indication that the rough-awned types are higher in starch and extract content than the smooth-awned types. However, in view of the comparatively small number of varieties under study and the anomalous behaviour of Nobarb, it is impossible to state definitely that differences exist between rough-awned and smooth-awned barleys with respect to their carbohydrate composition.

There is some evidence that the smooth-awned varieties tend to be lower in extract nitrogen. This is in agreement with the evidence presented in Part I (1) and Part V (2) that these barleys tend to be low in salt-soluble nitrogen and that the malts from them tend to be low in wort nitrogen.

Station Differences

The analytical results are summarized in Table II as means, over all varieties, for each station. The stations are listed in order of increasing nitrogen content of the barleys in order to facilitate examination of the relations between nitrogen and other constituents, a subject which is discussed in the next section.

TABLE II

MEAN VALUES FOR EACH STATION, FOR NITROGEN, STARCH, CRUDE CELLULOSE-LIGNIN, AND BARLEY EXTRACT

Station	Nitrogen, %	Starch, %	Crude cellulose-lignin, %	Extract, %	Extract nitrogen, %
Nappan	1.54	59.2	10.0	80.8	0.52
Fredericton	1.74	57.7	10.8	78.2	0.54
Ste. Anne de Bellevue	1.93	56.1	11.0	77.5	0.56
Ste. Anne de la Pocatière	2.28	54.2	10.9	76.8	0.62
Lethbridge	2.29	55.1	9.6	77.9	0.70
Winnipeg	2.33	53.6	10.2	74.7	0.64
Brandon	2.36	54.8	9.8	76.5	0.64
Guelph	2.38	52.4	10.6	74.6	0.68
Ottawa	2.53	53.3	9.9	75.5	0.68
Lacombe	2.67	52.7	10.0	74.4	0.70
Beaverlodge	2.67	51.7	10.4	73.6	0.67
Gilbert Plains	2.69	52.7	9.9	74.2	0.72
Mean, over all stations	2.28	54.5	10.3	76.2	0.64
Maximum spread	1.15	7.5	1.4	7.2	0.20
Necessary difference, 5% level	0.079	0.66	0.32	0.64	0.013

The data show that environmental differences resulted in the production of a series of samples showing fairly wide variations with respect to all properties measured. Moreover, comparison of the spreads between station means with the necessary difference required for a 5% level of significance, shows conclusively that environment has a significant effect on each of the various constituents. It is interesting to note that the maximum spreads between station means for starch and barley extract are somewhat greater than the corresponding spreads between varietal means (see Table I), whereas for the cellulose-lignin fraction the spread between varietal means is considerably greater than that between station means, i.e., 2.4% as against 1.4%. Since the cellulose and lignin in the barley kernels exist mainly in the hull, the data suggest that variety has a greater effect than environment on the total quantity and thickness of the hull. This assumption seems justified, since the data on which it is based were obtained by analyses of samples representing a very wide range of both varieties and environments.

TABLE III

ANALYSIS OF VARIANCE FOR BARLEY PROPERTIES: MEAN SQUARES

Barley property as % dry matter	Variance due to		
	Stations	Varieties	Interaction
Starch	61.474**	35.278**	0.649
Crude cellulose-lignin	2.563**	7.079**	0.151
Extract	54.650**	43.348**	0.612
Extract nitrogen	0.05531**	0.02105**	0.00108
Degrees of freedom	11	11	121

Correlations with Total Nitrogen

The correlations between total nitrogen and other constituents were studied by calculating the correlation coefficients for varietal and station means. The resulting statistics are given in Table IV.

TABLE IV

INTER-VARIETAL AND INTER-STATION CORRELATION COEFFICIENTS BETWEEN NITROGEN CONTENT AND BARLEY PROPERTIES

Barley property as % dry matter	Correlation coefficient	
	Inter-varietal	Inter-station
Starch	−.399	−.953**
Crude cellulose-lignin	.301	−.334
Extract	−.401	−.908**
Extract nitrogen	.241	.924**

Since none of the inter-varietal correlation coefficients are significant, the investigation yields no evidence of inter-varietal relations between total nitrogen and any of the other properties listed. On the other hand, several of the inter-station correlation coefficients are highly significant. With respect to station means, total nitrogen is significantly and negatively correlated with starch and extract and significantly and positively correlated with the nitrogen in the barley extract. Thus environmental conditions, which tend to increase total nitrogen, tend also to increase barley extract nitrogen, and to decrease starch and barley extract. These intra-varietal relations were certainly to be expected and no further discussion of them seems necessary.

Correlations with 1000-kernel Weight

The inter-varietal and inter-station correlation coefficients between 1000-kernel weight and other properties are given in the first two columns in Table V. Owing to the association between 1000-kernel weight and total nitrogen, it appeared that these statistics might reflect correlations with nitrogen, rather than actual correlations with 1000-kernel weight. The possible effects of nitrogen were therefore eliminated by calculating the partial correlation coefficients for 1000-kernel weight and each property, independent of total nitrogen. These correlation coefficients are listed in the last two columns of Table V. In general, the simple and partial coefficients are of the same order of magnitude, which indicates that nitrogen content has little or no influence on the relations.

TABLE V

INTER-VARIETAL AND INTER-STATION CORRELATION COEFFICIENTS BETWEEN 1000-KERNEL WEIGHT AND BARLEY PROPERTIES, AND PARTIAL CORRELATION COEFFICIENTS INDEPENDENT OF NITROGEN CONTENT

Barley property as % dry matter	Correlation coefficient		Partial correlation coefficient	
	Inter-varietal	Inter-station	Inter-varietal	Inter-station
Starch	.690*	.688*	.615*	.746**
Crude cellulose-lignin	−.621*	−.054	−.572	.149
Extract	.639*	.688*	.547	.605*
Extract nitrogen	−.300	−.552	−.206	−.221

Starch content and barley extract are positively correlated with 1000-kernel weight both within and between varieties. This is in agreement with the common assumption that barleys having larger kernels should yield a greater proportion of extractives due to a lower husk to endosperm ratio. Since the major portion of the cellulose and lignin occurs in the hulls of the barley, a negative association would be expected between this fraction and 1000-kernel weight. The exceptionally low value of the inter-station coefficient may be due to variations in the ash content of the residue from barley grown on different soil types. From the comparatively low values of all the correlation coefficients, it is apparent that 1000-kernel weight is a very poor indication of the potential extractives in a barley, and its importance in evaluating barley for malting purposes appears to have been over-emphasized.

No association exists between nitrogen content of the barley extract and 1000-kernel weight. This probably explains in part the lower degree of association between barley extract and kernel weight than between starch and kernel weight.

Correlations among the Carbohydrate Fractions

Correlation coefficients showing the interrelations of the various barley fractions under study are listed in Table VI. The first four columns contain the simple coefficients and the partial coefficients, independent of total nitrogen, for the inter-varietal correlations. The last four columns contain the corresponding data for inter-station correlations.

TABLE VI

CORRELATION COEFFICIENTS (SIMPLE AND PARTIAL, INDEPENDENT OF TOTAL NITROGEN) AMONG STARCH, EXTRACT, CELLULOSE-LIGNIN RESIDUE, AND EXTRACT NITROGEN OF BARLEY

Type of correlation	Independent variable	Inter-varietal coefficients			Inter-station coefficients		
		Cellulose-lignin	Barley extract	Extract nitrogen	Cellulose-lignin	Barley extract	Extract nitrogen
Simple	Starch	−.952**	.982**	.312	.028	.961**	−.859**
	Cellulose-lignin		−.968**	−.433		.027	−.498
	Barley extract			.074			−.749**
Partial, independent of total nitrogen	Starch	−.953**	.979**	.458	−.969**	.752**	.190
	Cellulose-lignin		−.971**	−.543		−.665*	−.557
	Barley extract			.355			.439

A very close positive association is shown to exist between starch content and barley extract both within and between varieties. This was to be expected since starch is the major constituent contributing to extract yield. But in addition to starch, other carbohydrates also contribute to extract and it appears that these other carbohydrate fractions must also be related to starch content. If barley extract is corrected for its nitrogen content the simple coefficients between starch and non-protein extract are 0.98 and 0.97 for inter-varietal and inter-station correlations. While these coefficients are slightly higher than those for total extract, it is evident that the extract nitrogen has an almost negligible influence on the starch-extract relations. These considerations suggest that in addition to a carbohydrate regularity within samples of the same variety (4), similar regularities exist for samples of different varieties grown under the same environmental conditions.

As would be expected, a highly significant negative association is indicated between the insoluble cellulose-lignin fraction and both starch and barley extract. These relations are perfectly clear-cut in the simple inter-varietal coefficients. They are, however, masked by common associations with total nitrogen in the inter-station simple coefficients but are readily proved to exist by calculating the partial coefficients independent of the effects of total nitrogen.

Within varieties, no relation appears to exist between the nitrogen content of the barley extract and the carbohydrate fractions. The simple interstation coefficients obtained between this property and both starch and barley extract are significant, but the partial coefficients independent of total nitrogen show clearly that these effects are due to common association of the carbohydrates and extract nitrogen with total nitrogen (cf. Table IV).

The most interesting feature of these relations is a regularity in the carbohydrate composition of barley (4) which appears to hold both within and between varieties. This is contrasted with a regularity in the protein composition which holds only within varieties (1). The partial correlation coefficients (cf. Table VI) and simple coefficients (cf. Table IV) indicate that within varieties there is an interdependence of the carbohydrate regularities on the nitrogen regularities, whereas between varieties no relations appear to exist between the nitrogen and carbohydrate contents of the barleys.

References

1. ANDERSON, J. A. and AYRE, C. A. Can. J. Research, C, 16 : 377-390. 1938.
2. ANDERSON, J. A., AYRE, C. A., and MEREDITH, W. O. S. Can. J. Research, C, 17 : 25-34. 1939.
3. BISHOP, L. R. J. Inst. Brewing, 40 : 75-91. 1934.
4. BISHOP, L. R. and MARX, D. J. Inst. Brewing, 40 : 62-74. 1934.
5. LÜERS, H. and MILLER, P. Wochschr. Brau. 52 : 153-154. 1935.
6. PAWLOWSKI, F. Die Brautechnischen, Untersuchungs-Methoden. R. Oldenbourg, München and Berlin: 35-39. 1932.
7. WAKSMAN, S. A. and STEVENS, K. R. Ind. Eng. Chem., Anal. Ed. 2 : 167-173. 1930

THE USE OF WATER BY WHEAT PLANTS WHEN INOCULATED WITH *HELMINTHOSPORIUM SATIVUM*[1]

By B. J. Sallans[2]

Abstract

The transpirational histories of wheat plants, inoculated with *Helminthosporium sativum* Pamm., King & Bakke to produce root rot, showed a marked reduction in water loss during the early stages of growth, when compared with uninoculated plants. This was accompanied by a corresponding reduction in the transpiring areas owing to reduced lengths and widths in the second, third, and fourth leaves to appear. As the plants entered into the early stages of elongation of the culm the transpirational story reflected a recovery in the inoculated plants. The later leaves to appear were longer with greater transpiring areas than in uninoculated plants. This fact combined with the death of the first three or four leaves resulted in greater transpirational and photosynthetic areas in the inoculated plants with consequent increased yields of dry matter. Probably similar recovery does not occur under competitive field conditions, where weeds and healthy wheat plants are present. Poor light conditions and low soil moistures were not conducive to recovery of inoculated plants. The water requirements of wheat did not appear to be affected significantly by inoculation. Of two varieties of spring wheat, Reward was more severely injured initially and recovered more rapidly than Marquis. Several possible explanations of the recovery recorded here are discussed.

Introduction

A disease of wheat-stem bases and roots caused by *Helminthosporium sativum* Pamm., King & Bakke is of widespread occurrence in Western Canada. It is one of several diseases affecting the underground portions of the stems, the crown, the subcrown internodes, and roots, which have similar symptoms and which collectively are known as "common root rot" or "foot rot". This disease has several manifestations such as pre-emergence blight, seedling blight, tiller blight, and prematurity blight. However, by far the most important phase of the disease under conditions in Western Canada is one in which the underground parts of the plants become lesioned with external and internal discolorations of the tissues, the plants being more or less stunted and their yields of grain reduced; but a phase in which the plants are not usually killed outright.

The injury to the plants and the reduction in yield of grain are difficult to estimate under these conditions. The diseased plants seldom occur in definite patches, but rather as individuals scattered among healthy or slightly diseased plants. Thus, it is not possible, ordinarily, to compare yields of affected areas of a field with yields of unaffected parts, as may be done with certain other root rots of cereals such as take-all (*Ophiobolus graminis* Sacc.) of wheat. While the author (8) has found that individual plant yields may be reduced

[1] *Manuscript received November 14, 1939, and as revised March 13, 1940.*

Contribution No. 606 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Wisconsin.

[2] *Assistant Plant Pathologist, Dominion Laboratory of Plant Pathology, Saskatoon, Saskatchewan.*

by 30 to 45% coincident with the presence of severe subcrown internode lesions, as compared with unlesioned or slightly lesioned neighbouring plants, the problem of competition for soil moisture and nutrients complicates the determination of actual net losses in yield per unit area of land.

Under the prevailing climatic conditions of Western Canada, soil moisture may be considered the most important limiting factor in wheat production. Any economy or wastage of soil moisture, as influenced by the practice of summerfallow or the growth of weeds, generally has an important effect upon the following cereal crops. The influence of *H. sativum* upon the transpiration of water by wheat plants when the underground parts are attacked was considered a necessary study in an evaluation of the disease.

It is well known that certain foliage diseases decrease or accelerate the transpiration of water by plants. A number of papers, of which Bever's (1) is a recent example, show that cereal rusts in particular increase water loss. The influence of parasites of underground portions of the plant, however, seems to have received little attention. Burkholder (2) reported transpiration studies on bean plants infected by the root parasite, *Fusarium Martii* f. *Phaseoli* Burkh. In the uninoculated series the water requirement increased with increased soil moistures. There appeared to be reduced water requirements in the inoculated plants, especiallly at the lower soil moistures. The transpirational data indicate very much reduced use of water by the inoculated plants, this being most evident in dry soil and less so in medium and wet soil. Linford (4) studied the transpirational history of peas infected by the *Fusarium* wilt organism. He found a decreasing amount of transpiration in diseased plants up to the time when wilting became evident. Most plants then showed a slightly increasing rate followed in a few days by a rapid decrease when wilting was complete.

In the present work data were taken in such a way as to secure transpirational histories of plants inoculated with *H. sativum* as compared with uninoculated plants, in addition to information on water requirements.

Since considerable differences were observed in the amounts of water transpired by plants owing to the presence or absence of root rot, it was thought that a study of transpiring areas might yield interesting information. Considerable stunting was observable in the seedling stage of inoculated plants. Yet under the conditions of some of the experiments there was an evident recovery in size, further indications of which were given by the data on water transpired per plant. Leaf areas were measured in Experiments III and IV.

The results reported in this paper were obtained under the artificial conditions of the greenhouse in winter and are not strictly applicable in the interpretation of water relations in the field.

Methods

The gravimetric method was adopted to measure transpirational losses. Regular weighings were made at intervals of two days to secure consecutive data from the early seedling stage to maturity, and losses were made up by

adding water from a burette. The pots or crocks were regularly and systematically interchanged on the greenhouse bench to compensate for environmental differences.

Of the various methods available for measuring transpiring areas, the one selected involved direct measurements and calculation. The leaves could thus be measured at any time during their functional period without removal from the plants. Leaf blade areas were calculated from the leaf length measured to the nearest millimetre, the greatest width estimated to the nearest tenth of a millimetre, and a factor. The factor was determined by measuring the widths of the leaf at one-centimetre intervals along its length. The mean width was then multiplied by two and divided by the maximum width to give the desired factor. The factor obtained and its standard deviation were 1.611 ± 0.013. Inoculation, soil moistures, and varietal differences did not significantly affect the factor. The first foliage leaf differs in shape from succeeding ones and 1.84 was the factor determined for it. In addition to the areas of leaf blades the exposed areas of culms and leaf sheaths were estimated.

Some sets of data were examined statistically by Fisher's analysis of variance method as outlined by Goulden (3).

Experiment I

A preliminary experiment was started in the greenhouse at Saskatoon, Saskatchewan, on February 17, 1936. Sixteen crocks of one Imperial gallon were used. The soil was not sterilized. Four crocks were seeded to Marquis and four to Reward wheat as controls. Similarly, four crocks were seeded to each variety, with seed that had been inoculated with conidia and mycelium of *H. sativum*. In addition, five crocks were prepared in the same manner but left unseeded. Water was added to bring the soil moisture to 50% of the moisture-holding capacity of the soil. The amount of water lost by transpiration was estimated by subtracting the average amount of water lost by evaporation from the five unplanted crocks from the total amount lost from each planted crock.

On Feb. 27 the plants that emerged were thinned to a uniform stand of 36 per crock. This was done by first removing excessively stunted and blighted seedlings and then removing at random the seedlings in excess of 36. On March 13 a further reduction to 12 per crock was made, again at random. The 24 plants removed from each crock were used to determine dry weight per plant. By considering the removed seedlings as samples of the whole population of 36 plants, estimates of the water requirements of the seedlings were made. These data are given in Table I and the analyses of variance in Table II.

While the dry weights per plant were significantly reduced by inoculation with *H. sativum*, there were corresponding reductions in the amounts of water transpired, hence no significant differences in water requirements due to inoculation were obtained. The interaction IU $\times$ Variety is significant for

TABLE I

DATA AT THE SEEDLING STAGE IN EXPERIMENT I

Variety	Treatment	Dry weight of plants, gm.	Water transpired, gm.	Water requirement, gm.
Marquis	Uninoculated	2.21	1162	350
Marquis	Inoculated	2.00	1017	346
Reward	Uninoculated	2.30	1190	344
Reward	Inoculated	1.73	783	323

TABLE II

ANALYSES OF VARIANCE OF DATA SUMMARIZED IN TABLE I

Source of variance	D.f.	Mean variances		
		Dry weight of plants	Water transpired	Water requirements
Replicates	3	0.0381**	11975	350.7
Treatments	3	0.2602**	138968**	581.6
IU	1	0.6123**	305533**	637.6
Variety	1	0.0333*	42333*	826.6
IU × Variety	1	0.1350**	69037**	280.6
Error	9	0.0043	5251	193.6

*Throughout this paper, "IU" will be used in analyses of variance tables for "inoculated" and "uninoculated". The 5% and 1% levels of significance will be designated by * and ** respectively.*

dry weights of plants and water transpired. It appears that Reward was more susceptible than Marquis to injury from the fungus in this experiment.

The experiment was continued to maturity of the plants, when yields and disease ratings were obtained. Table III gives in condensed form the transpirational histories of plants in the various treatments. The water losses are given for 11 periods of 9 and 10 days each.

The relation of the amount of water used by the inoculated plants to that used by the uninoculated plants is graphically represented in Fig. 1. The

TABLE III

THE WATER LOSS BY TRANSPIRATION IN GRAMS PER PLANT IN EXPERIMENT I

Variety	Treatment	Periods										
		1	2	3	4	5	6	7	8	9	10	11
Marquis	Uninoculated	14	35	53	70	96	103	120	118	95	101	24
Marquis	Inoculated	11	34	53	77	106	110	126	122	96	103	25
Reward	Uninoculated	14	35	58	82	111	105	111	96	42	10	0
Reward	Inoculated	8	29	49	77	116	115	122	108	58	18	1

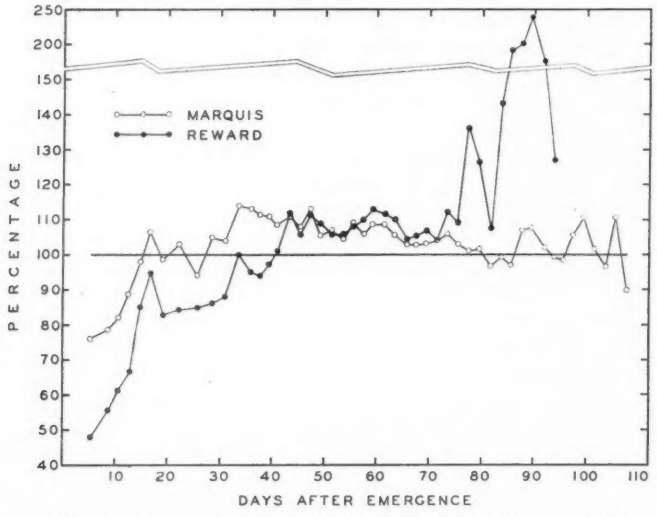


FIG. 1. *Graphs representing transpirational histories in Experiment I of Marquis and Reward wheats inoculated with Helminthosporium sativum, expressed as percentages of their respective uninoculated controls. The horizontal straight line at the 100% mark represents the controls. The data were recorded to maturity of the control plants.*

figure was prepared from data for periods of 2 or 4 days in the seedling stage and of 2 days thereafter. In Table III these data were condensed to fewer periods. The total estimated transpiration, for the four replicates of each variety inoculated with *H. sativum*, is expressed as a percentage of the corresponding figure for uninoculated plants. The initial low transpiration by inoculated plants, especially in Reward, was followed by a period of recovery, following which the inoculated plants used more water than the controls. The Reward control matured about four days before the inoculated plants, hence the greatly increased ratio in use of water by the latter toward the end of the experimental period.

Yields of grain, total dry weight of tops, and the total amount of water used per plant are given in Table IV. Water requirements in grams for each gram of oven-dry matter were calculated. Table V gives the analyses of variance of these data.

The yield of grain, the total dry plant weights, and the total water used by inoculated plants were significantly higher than in the uninoculated plants. The inoculated plants appeared to be significantly more economical in the use of water than the controls. The non-significance of the interaction Variety × IU in any of the sets of data indicates that Marquis and Reward responded similarly to inoculation.

The analysis of the average weight per seed data shows no difference ascribable to variety or inoculation with *H. sativum*. There are, however, significantly influenced numbers of seeds per plant due to treatments. The

TABLE IV

YIELDS OF GRAIN, DRY WEIGHT OF PLANT TOPS, WATER USED PER PLANT, AND WATER REQUIREMENTS IN EXPERIMENT I

Variety	Treatment	Yield of grain		Weight per seed, mg.	Dry weight of plant tops, gm.	Total water used per plant, gm.	Water requirements, gm.
		Weight, gm.	Seeds, no.				
Marquis	Uninoculated	9.1	261	35.3	27.5	829	361
Marquis	Inoculated	9.8	283	34.7	30.2	863	343
Reward	Uninoculated	9.4	273	34.5	25.3	664	315
Reward	Inoculated	10.9	321	34.0	28.3	701	297

TABLE V

ANALYSES OF VARIANCE OF THE DATA SUMMARIZED IN TABLE IV GIVING THE MEAN VARIANCES

Source of variance	D.f.	Mean variances					
		Weight of grain	No. of seeds	Weight per seed	Dry weight of plant tops	Total water used per plant	Water requirement
Replicates	3	0.11	17	2.32	0.92	532	21.7
Treatments	3	2.48**	2683**	1.22	16.72**	37199**	3269.0**
Variety	1	1.96**	2500**	2.40	16.40**	106439**	8418.0**
IU	1	4.84**	4900**	1.21	33.64**	5148**	1387.5**
Variety × IU	1	0.64	650	0.04	0.12	11	1.5
Error	9	0.14	169	0.83	0.57	422	121.1

increased yields in the inoculated plants are not due, therefore, to kernels of different weight, but to the numbers of kernels produced by each plant. Thus, it appears that any influence exerted by inoculation with *H. sativum* on yield occurred before seed was set and not during the period of filling of the grain.

Experiment II

The second experiment was started in the greenhouse at the University of Wisconsin on November 18, 1936. No artificial light was supplied, consequently the plants grew poorly during the period of short days. Reward finally headed, but Marquis remained in a vegetative condition until the experiment was terminated on March 20. Under these conditions there was no marked tendency for the inoculated plants to recover, indicated in the transpirational data. Since the results of this experiment are in entire agreement with those of Experiment IV, in so far as they may be compared, the data need not be given here.

Experiment III

The third experiment, conducted at the University of Wisconsin, was started February 13, 1936, in a 20° C. greenhouse. Four levels of soil moisture were used, namely 30, 45, 60, and 75% of the M.H.C. of the soil. One variety of wheat, Reward, was sown, the seed for one-half the containers at each moisture level being inoculated with *H. sativum*. As the experiment was conducted in quadruplicate there were 32 containers.

The data on transpiration were assembled into nine periods. The first eight periods consist of ten days each, and the ninth period of six days. The plants were for the most part fairly well matured when harvested on May 26. The data are given in Table VI.

TABLE VI

TRANSPIRATIONAL DATA FOR EXPERIMENT III

M.H.C. of soil, %	Treatment	Periods								
		1	2	3	4	5	6	7	8	9
75	Uninoculated	642	1585	2423	2899	3237	2108	2491	1124	110
75	Inoculated	472	1087	2032	3058	3832	2723	3296	1949	559
60	Uninoculated	619	1354	2045	2621	3157	2486	3083	1955	445
60	Inoculated	415	989	1948	2814	3566	2881	3652	2634	820
45	Uninoculated	391	870	1022	1130	1467	1361	1817	1419	562
45	Inoculated	227	587	907	835	999	920	1305	1147	506
30	Uninoculated	73	282	508	586	636	523	665	498	160
30	Inoculated	74	180	368	504	476	397	492	401	163

The water transpired in the early periods is small compared to that transpired by the full-grown plants, and a pooled estimate of variance cannot be used in evaluating main effects and interactions involving periods. To overcome this difficulty the data were transformed to logarithms to minimize the contrasts between periods. Williams (11) has made a similar use of logarithms in analysing entomological data.

To test whether the error variances were markedly different from period to period in the transformed data, use was made of the method of Yates and Cochran (12) in determining whether error mean variances of the same experiment at different stations and in different years were in fact the same. The error variances for the periods were sufficiently alike to be considered the same. Table VII gives the mean logarithms of the data summarized in Table VI, and Table VIII the analysis of variance of the transformed data.

The mean variances for periods and moistures are highly significant. The amount of water transpired by plants grown at the higher soil moistures is greater than at lower soil moistures due to the larger leaf areas in part and to the greater ease with which water may be withdrawn from the soil. The mean variance for IU is not significant. This is due to the greater losses by transpiration at 60 and 75% M.H.C. in the inoculated series being counterbalanced largely by the relatively smaller losses at 30 and 45%, as shown in

TABLE VII

MEAN LOGARITHMS OF DATA SUMMARIZED IN TABLE VI

M.H.C. of soil, %	Treatment	Periods								
		1	2	3	4	5	6	7	8	9
75	Uninoculated	2.803	3.199	3.383	3.461	3.509	3.322	3.395	3.048	1.971
75	Inoculated	2.671	3.035	3.304	3.480	3.580	3.431	3.515	3.287	2.737
60	Uninoculated	2.787	3.122	3.303	3.412	3.495	3.394	3.488	3.287	2.631
60	Inoculated	2.616	2.993	3.285	3.447	3.550	3.459	3.561	3.419	2.903
45	Uninoculated	2.582	2.912	2.974	3.020	3.151	3.128	3.258	3.151	2.735
45	Inoculated	2.351	2.761	2.948	2.897	2.973	2.937	3.097	3.041	2.694
30	Uninoculated	1.848	2.450	2.706	2.764	2.798	2.707	2.818	2.693	2.197
30	Inoculated	1.866	2.252	2.564	2.700	2.661	2.582	2.675	2.595	2.212

TABLE VIII

ANALYSIS OF VARIANCE OF LOGARITHMS OF DATA SUMMARIZED IN TABLE VI

Source of variance	Sum of squares	D.f.	Mean square
Replicates	0.036157	3	0.012052
Treatments	24.299005	7	3.471286**
Moistures	23.581827	3	7.860609**
IU	0.037721	1	0.037721
Moistures × IU	0.679457	3	0.226486**
Error I	0.890425	21	0.042401
Periods	22.517679	8	2.814710**
Periods × moistures	2.858726	24	0.119113**
Periods × IU	0.903290	8	0.112911**
Periods × moistures × IU	0.790471	24	0.032936**
Error II	1.196329	192	0.006231

Table IX. The highly significant interaction, Moistures × IU, leads to the conclusion, however, that inoculation does affect transpiration, and that the effects vary with different levels of soil moisture. At 60 and 75% M.H.C. inoculation accelerated transpiration, while at 30 and 45% M.H.C. inoculation retarded transpiration.

The interaction, Periods × IU, is highly significant. Fig. 2 indicates that transpiration in the inoculated plants was low in the early periods but at later periods tended to equal and even surpass that of the uninoculated plants. The second order interaction, Periods × Moisture × IU, which is also significant statistically, indicates that plants at all moistures did not respond in the same way to inoculation at all periods. This is clearly seen in Fig. 2, where the curves for transpiration in the inoculated plants at 30 and 45% M.H.C. do not rise above the uninoculated straight line curve as do those at 60 and 75% M.H.C. in the later periods. In this figure it appears that inoculated plants at 30 and 45% M.H.C. recovered at a slower rate than those at the higher moisture levels, but at about the period of maximum growth

the tendency toward recovery was lost, and the relative transpirational curves quickly reverted to approximately their original levels. The high portion of the curve for inoculated plants at 30% M.H.C. between 10 and 20 days after emergence is due to a wilted condition in the controls at this moisture level, which was much more pronounced than in inoculated plants.

Data on oven-dry weights of plants including roots, and the total water transpired were used to calculate the water requirements. These three sets of data are shown in Table IX. Table X gives the analyses of variance.

TABLE IX

DATA FROM EXPERIMENT III ON DRY WEIGHTS OF PLANT TOPS AND TOTAL WATER USED, AND WATER REQUIREMENTS

M.H.C. of soil, %	Treatment	Yield of dry matter, gm.	Water used per pot, gm.	Water requirements, gm.
75	Uninoculated	25.87	16619	643
75	Inoculated	29.07	19008	654
60	Uninoculated	33.72	17765	526
60	Inoculated	37.22	19719	529
45	Uninoculated	23.92	10039	421
45	Inoculated	19.35	7433	401
30	Uninoculated	11.02	3931	355
30	Inoculated	8.50	3055	358

TABLE X

SUMMARY OF ANALYSES OF VARIANCE ON THE THREE SETS OF DATA OF TABLE IX

Source of variance	D.f.	Mean square		
		Yield of dry matter	Water used	Water requirements
Replicates	3	6.089	1118925	1595.5
Treatments	7	421.123**	190191371**	57958.3**
Moistures	3	942.656**	432392608**	134871.8**
IU	1	0.980	369800	5.3
Moistures × IU	3	39.637*	11263658*	362.4
Error	21	10.938	2820465	664.8

The yields of dry matter and the amounts of water transpired were not influenced appreciably, as shown by the mean variances for inoculation with *H. sativum*. However, an examination of Table IX indicates different effects produced by inoculation at the different levels of soil moisture. Furthermore, the interaction of moistures and inoculation gives values that lie between the 5% and 1% levels of significance. Thus, it appears that yields of dry matter and water transpired were significantly increased by inoculation at the higher levels of soil moisture, and significantly reduced at the lower levels. The water requirement was very greatly influenced by soil moisture levels, but

inoculation had no significant effect in this experiment. No significance can be attached to the interaction effect here, indicating that the interaction effects observed for yield of dry matter and total water consumption are closely related.

Transpirational area measurements were made at three stages of development of the plants in this experiment. Three plants were selected at random for these records. Measurements were made on March 8 and 24, and May 9. By the latter date a number of the lower leaves had dried up. They varied from five to six at the lower soil moisture levels to about seven or eight at the 75% soil moisture level.

The total water losses for periods of six days, centering on each of the dates of measurement, were taken from the records of transpiration, given in summarized form in Table VI. From these and data on transpiring areas (Table XI) the rates of water loss were calculated (Table XII). The data on rates of transpiration were subjected to analysis of variance (Table XIII).

Table XI shows the greatest reduction in areas at the first date of measurement. Expressing the areas of the inoculated plants as percentages of the areas of uninoculated plants regardless of soil moistures, they are 56, 71,

TABLE XI

TRANSPIRING AREAS PER PLANT

M.H.C. of soil, %	Treatment	Transpiring areas, sq. cm.		
		March 8	March 24	May 9
75	Uninoculated	65	262	151
75	Inoculated	39	224	210
60	Uninoculated	62	303	262
60	Inoculated	36	204	279
45	Uninoculated	42	191	128
45	Inoculated	19	114	123
30	Uninoculated	19	72	96
30	Inoculated	12	51	94

TABLE XII

RATE OF TRANSPIRATION IN EXPERIMENT III

M.H.C. of soil, %	Treatment	Mg. per sq. dm. per hr.		
		March 8	March 24	May 9
75	Uninoculated	462	320	626
75	Inoculated	591	284	630
60	Uninoculated	471	238	464
60	Inoculated	547	325	503
45	Uninoculated	443	221	572
45	Inoculated	549	284	457
30	Uninoculated	182	237	297
30	Inoculated	272	235	218

TABLE XIII

ANALYSIS OF VARIANCE OF RATES OF TRANSPIRATION IN TABLE XII

Sources of variance	D.f.	Mean squares
Replicates	3	14528
Treatments	7	120634**
Moisture	3	269748**
IU	1	21570
Moistures × IU	3	4542
Error I	21	6420
Periods	2	381579**
Periods × Moistures	6	46076**
Periods × IU	2	37811**
Periods × Moistures × IU	6	6608
Error II	48	4925

and 111% for the three dates, respectively. On May 9 the areas of inoculated plants at 60 and 75% M.H.C. of the soil were greater than in the uninoculated plants, while at 30 and 45% M.H.C. they were slightly less. These facts are correlated with those shown by the transpirational histories in Table VI and Fig. 2.

Combining the data on areas with water loss to derive rates of transpiration as in Table XII, however, it becomes evident that the intensity of transpiration varies with the different treatments. In addition to the obvious effects of periods there are highly significant effects due to soil moistures and the inter-

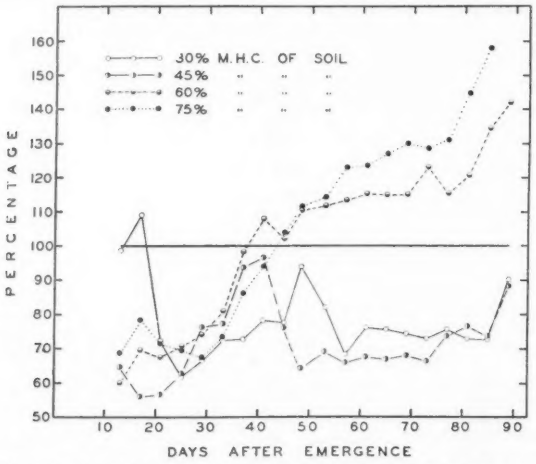


FIG. 2. *Transpirational histories of wheat inoculated with Helminthosporium sativum. Water losses over four-day periods are shown as percentages of the water losses of uninoculated plants. The horizontal straight line at the 100% point represents the water used by the controls. Comparisons are shown for soil moisture maintained at 30, 45, 60, and 75% M.H.C. of the soil.*

action of periods with moistures. Inoculation significantly increased the rate of transpiration during the first period. This effect was less with each succeeding period, resulting in the significant interaction, Periods × IU.

The average areas of the successively appearing leaves indicate the mechanism by which inoculated plants produced greater yields than the controls. In Fig. 3 the average areas of leaves of inoculated plants (black bars) are compared with those of control plants. Inoculation very markedly reduced

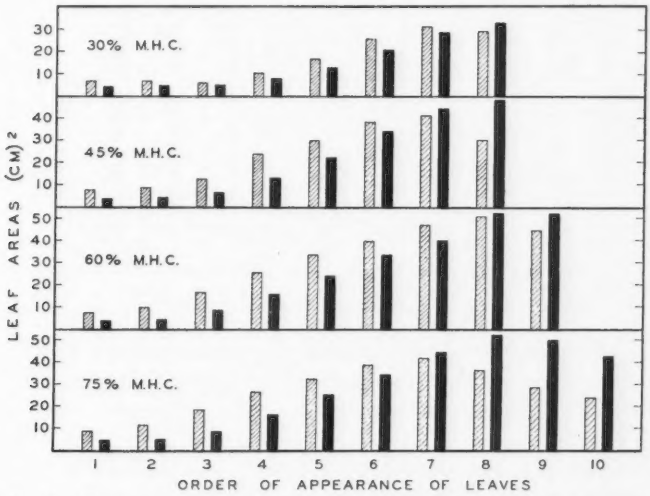


FIG. 3. *Bar diagrams showing transpiring areas in square centimetres of leaves on the main culms, in their order of appearance from inoculated wheat plants (black bars) compared with those from uninoculated plants (diagonally marked bars). The comparisons are made at soil moisture levels of 30, 45, 60, and 75% M.H.C. of the soil.*

the areas of the leaves up to about the sixth or seventh to appear. The later leaves to appear were larger in the inoculated plants, particularly at 75% M.H.C. As the earlier leaves died successively the total transpiring areas in the inoculated plants became more nearly equal to those of uninoculated plants, and eventually became greater as the process went on. Thus, during the flowering and filling stages the products of photosynthesis in the inoculated plants at the higher soil moistures were probably greater than in the control, accounting for increased yields. At the lower soil moistures the same factors were at work. However, the recovery was not sufficiently rapid to show the same beneficial results in increased yields. In Fig. 3 the difference between inoculated and controls at 60% M.H.C. will not account for the increased yields in the inoculated plants. In this case more tillers and more main culm leaves in the inoculated plants were sufficient to account for the increased yields.

Fig. 4 was prepared from the lengths and widths of the leaves of the main culms to show how the leaf areas were first reduced and then increased in

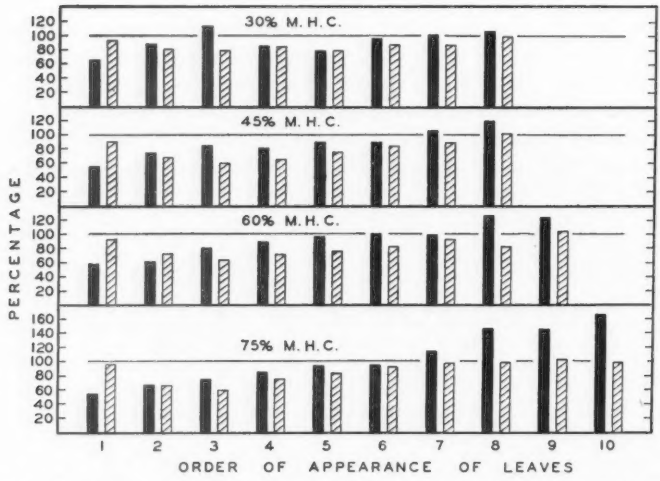


FIG. 4. *Bar diagrams showing leaf lengths (black bars) and leaf widths (diagonally marked bars) of wheat inoculated with Helminthosporium sativum as percentages of leaf lengths and widths of control-plant leaves. The plants were grown at four levels of soil moisture.*

the inoculated plants compared with the controls. The black bars represent leaf lengths and the diagonally marked bars represent leaf widths of the inoculated plants expressed in percentage of the lengths and widths of control plant leaves.

The first leaf to appear was reduced in width very slightly by inoculation, but it was reduced in length to 60% or less of the control. Successive leaves in the inoculated plants improved in length until the later ones exceeded those of the controls. In width the leaves of inoculated plants dropped from about normal in the first leaf to about 60% of normal in the third leaf. In succeeding leaves width improved to normal. However, it is interesting to note that the increased areas of the later leaves of inoculated plants (Fig. 3) were due entirely to increased lengths, while the widths remained as in the controls.

Experiment IV

This experiment was conducted at Saskatoon from December 12, 1937, to March 24, 1938. The soil was autoclaved at 15 lb. pressure for 2 hr. The treatments were as in Experiment III. Evaporation from the soil was largely prevented by covering the surface with a mixture of fine sand and petrolatum. This was packed firmly around the bases of the plants and edges of the containers. The seedlings were thinned to three in each pot. Each replicate was placed in a block in a permanent position on the greenhouse bench. The four blocks were arranged in a 2 × 2 square. At each weighing the positions of pots within the blocks were randomized. Since the experiment was begun in a period of short days, artificial light was supplied from 5 p.m. to 8 a.m.

throughout the course of the experiment. This added light was insufficient, however, to produce good growth.

The transpirational data were assembled into ten periods of ten days each, and are presented in Table XIV. An analysis of variance is given in Table XV.

TABLE XIV

DATA ON TRANSPIRATION FOR EXPERIMENT IV

M.H.C. of soil, %	Treatment	Periods									
		1	2	3	4	5	6	7	8	9	10
75	Uninoculated	19	48	110	180	217	240	299	252	233	185
75	Inoculated	12	31	80	131	173	209	206	187	150	144
60	Uninoculated	18	39	79	124	142	182	210	204	186	141
60	Inoculated	12	29	67	112	132	177	202	169	162	122
45	Uninoculated	11	22	38	59	74	116	111	86	64	41
45	Inoculated	8	14	28	50	66	96	89	82	61	31
30	Uninoculated	11	15	18	25	26	31	21	15	10	0
30	Inoculated	8	13	16	25	25	32	24	16	11	0

TABLE XV

ANALYSIS OF VARIANCE OF LOGARITHMS OF DATA SUMMARIZED IN TABLE XIV

Source of variance	Sums of squares	D.f.	Mean squares
Replicates	1.412803	3	0.470934**
Treatments	47.844885	7	6.834983**
Moistures	47.137578	3	15.712526**
IU	0.548633	1	0.548633**
Moistures × IU	0.158673	3	0.052891
Error I	0.562024	21	0.026763
Periods	28.006400	9	3.111822**
Periods × Moistures	14.528706	27	0.538100**
Periods × IU	0.130970	9	0.014552
Periods × Moistures × IU	0.041686	27	0.001544
Error II	3.180747	216	0.014726

The results of this experiment are comparable with those of Experiment II, in which light conditions were unfavourable for good growth of the plants also. The mean variance for IU is highly significant while those for Periods × IU and the second order interaction are non-significant. These facts indicate that inoculation was effective in reducing transpiration, and that there was no marked tendency under the conditions of the experiment for inoculated plants to recover.

Oven-dry weight of plants including the roots and the total water transpired are given in Table XVI. From these two sets of data the water used per gram of dry matter was calculated for each unit of the test. These data also are presented in Table XVI. Table XVII shows the analyses of variance.

TABLE XVI

DATA FROM EXPERIMENT IV ON DRY WEIGHTS OF PLANTS, TOTAL WATER USED PER CONTAINER AND WATER REQUIREMENTS

M.H.C. of soil, %	Treatment	Yield of dry matter, gm.	Water used per container, gm.	Water requirements, gm.
75	Uninoculated	2.84	1783	638
75	Inoculated	1.96	1323	678
60	Uninoculated	2.23	1325	604
60	Inoculated	1.82	1184	654
45	Uninoculated	1.02	622	612
45	Inoculated	0.86	525	614
30	Uninoculated	0.86	172	453
30	Inoculated	0.38	170	456

TABLE XVII

ANALYSES OF VARIANCE OF DATA SUMMARIZED IN TABLE XVI

Source of variance	D.f.	Mean variances		
		Yield of dry matter	Water used per container	Water requirements
Replicates	3	0.7628**	165887**	5276*
Treatments	7	3.3070**	1427772**	29843**
Moistures	3	7.0669**	3170783**	66898**
IU	1	1.0658**	245350**	4465
Moistures × IU	3	0.2942	78901	1247
Error	21	0.1084	29981	1584

While the total water used per container was markedly affected in this experiment by inoculation with *H. sativum*, the water requirement was not significantly influenced. The interaction, Moisture × IU, was not significant either for yield of dry matter or for total water transpired per container. Thus, it appears that in this experiment, conducted under poor light conditions, there was no tendency at the higher soil moisture levels for inoculated plants to produce as much dry matter as uninoculated plants.

Transpiring areas were calculated from measurements made on all leaves and stems of all plants in this experiment on December 24, January 5, 17, and 31. Table XVIII shows the average transpiring areas per plant for the various treatments at the four dates of determination in square centimetres.

The total water transpired from each flask for eight-day periods centering on each of these dates was used to calculate the rates of transpiration. These are given in Table XIX and the analysis of variance in Table XX.

As in Experiment III the transpirational areas of inoculated plants are reduced to 60% or less of those in the uninoculated plants, at the first time of measurement. However, unlike Experiment III there was only slight

TABLE XVIII

TRANSPIRING AREAS (SQ. CM.) PER PLANT, IN EXPERIMENT IV

M.H.C. of soil, %	Treatment	Dec. 24	Jan. 5	Jan. 17	Jan. 31
75	Uninoculated	18.8	46.8	72.3	98.0
75	Inoculated	10.8	27.0	51.4	63.0
60	Uninoculated	19.0	41.6	61.3	66.4
60	Inoculated	11.2	29.7	52.1	62.2
45	Uninoculated	16.6	36.1	47.3	44.4
45	Inoculated	9.6	20.3	34.1	32.6
30	Uninoculated	16.5	30.9	31.1	21.6
30	Inoculated	9.9	20.1	30.2	19.6

TABLE XIX

RATE OF TRANSPIRATION IN EXPERIMENT IV

M.H.C. of soil, %	Treatment	Mg. per sq. dm. per hr.			
		Dec. 24	Jan. 5	Jan. 17	Jan. 31
75	Uninoculated	203	241	307	262
75	Inoculated	219	269	299	317
60	Uninoculated	168	196	253	242
60	Inoculated	198	221	271	252
45	Uninoculated	120	110	163	198
45	Inoculated	136	142	188	242
30	Uninoculated	110	78	101	146
30	Inoculated	139	99	104	159

TABLE XX

ANALYSIS OF VARIANCE OF RATES OF TRANSPIRATION, TABLE XIX

Source of variance	D.f.	Mean squares
Replicates	3	624.0
Treatments	7	61221.7**
Moistures	3	137288.4**
IU	1	15975.8**
Moistures × IU	3	236.9
Error I	21	842.0
Periods	3	32149.3**
Periods × Moistures	9	4626.3**
Periods × IU	3	638.8
Periods × Moistures × IU	9	461.1
Error II	72	398.0

recovery in succeeding periods. This agrees with the transpirational histories (Table XIV), in which the failure of the inoculated plants to recover fully, even at the higher soil moistures, was attributable to poor light conditions.

Rates of transpiration, Table XIX, are significantly greater in the inoculated than in the uninoculated plants as in Experiment III. On the other hand the non-significant interaction, Periods × IU, indicates the failure of the plants to recover. There was an average of 12% more water transpired per unit area by inoculated plants than by uninoculated plants. This varied only slightly from period to period and from one soil moisture level to another, yet the 4% increase in the water requirements due to inoculation failed to reach the level of significance. There may have been an increased production of dry matter per unit of leaf area which kept down the water requirements in spite of the increased rate of transpiration.

Experiment V

This experiment, conducted at Madison, Wis., was designed to determine whether inoculated seedlings had higher or lower water requirements than uninoculated seedlings. The data are summarized in Table XXI and the analyses of variance are given in Table XXII.

TABLE XXI

DRY WEIGHTS OF TOPS AND ROOTS OF WHEAT IN EXPERIMENT V, WEIGHT OF WATER TRANSPIRED AND WATER REQUIREMENTS

M.H.C. of soil, %	Treatment	Dry weight, mg.		Water transpired per plant, gm.	Water requirements, gm.
		Tops	Roots		
75	Uninoculated	68.2	10.8	46.6	590
75	Inoculated	64.2	10.6	46.1	616
60	Uninoculated	77.2	12.6	48.7	541
60	Inoculated	62.2	10.8	40.4	555
45	Uninoculated	63.2	15.8	32.9	417
45	Inoculated	51.4	13.4	25.8	399
30	Uninoculated	42.4	17.4	12.6	211
30	Inoculated	38.2	14.2	13.8	262

TABLE XXII

ANALYSES OF VARIANCE OF DATA SUMMARIZED IN TABLE XXI

Source of variance	D.f.	Mean squares			
		Dry weight		Water transpired per plant	Water requirements
		Tops	Roots		
Replicates	4	134.1	12.79	116.74*	2094
Treatments	7	881.2**	31.49**	1063.66**	113965**
Moistures	3	1724.5**	57.40**	2386.01**	262234**
IU	1	765.6**	36.10*	129.24	4452
Moistures × IU	3	76.4	4.03	52.79	2201
Error	28	58.9	6.96	41.19	1218

While significant reductions in dry weight of tops and roots of the seedlings were produced by *H. sativum*, particularly at 60 and 45% M.H.C. of the soil, the water transpired and the water requirements were not significantly influenced. The increase in water requirements due to inoculation at 30% M.H.C. is explainable by the fact that wilting was apparent in the control seedlings at this moisture level two to three weeks after emergence. Root development at the lower soil moisture levels was significantly greater than at the higher.

Discussion

The central fact brought out in this work is the recovery of plants inoculated with *H. sativum* after an initial severe set-back in the seedling stage. Inoculation of the seed with the fungus before sowing ensured an immediate attack on the seedlings during and immediately following germination. A retardation in emergence of one to two days occurred in all experiments. Rates of transpiration, leaf areas, dry weights, and disease ratings all indicated a poor development of the inoculated seedlings. The inoculated seedlings were reduced to as low as 50% or less of the controls in leaf areas and transpiration.

Under favourable conditions the retarded seedlings showed definite signs of recovery as indicated by the transpirational curves in relation to the controls and by relatively increasing sizes in successively appearing leaves. That the recovery is more than mere catching up to the controls is indicated by an actually greater use of water, greater leaf areas, and increased yield in the inoculated as compared with control plants.

That conditions of growth must be favourable to the plant before recovery can occur is indicated by Experiments II and IV, in which light conditions were poor, and by Experiment III, in which recovery was permanent only at the higher soil moisture levels. Slow recovery appeared in the latter experiment at 30 and 45% M.H.C. of the soil. At about the time of elongation of the culms, however, the tendency to recover was suddenly lost as indicated by the transpirational history, and the plants reverted to about the same level as in the seedling stage, in their relative use of water.

The recovery of the inoculated plants from the initial set-back was attended by a more rapid appearance of leaves, the formation of more leaves, and greater areas in the later appearing leaves. As the plants approached the flowering and later stages, the lower leaves of both inoculated and uninoculated plants died in succession. With the loss of the lower stunted leaves, the total functional leaf areas of the inoculated plants became greater than in the controls. The greater photosynthesis in consequence, would account for the increased yields that were observed.

The initial set-back following infection of wheat by *H. sativum* may be due to a toxin, in view of the findings of Vanterpool (10). He reports that *H. sativum* grown on wheat-grain medium produced a water-soluble toxin that caused slight retardation of the plumules and moderate shortening of the roots in germinating wheat. What the influence of the toxin may be on the plant to cause stunting is obscure. Possibly the formation of growth promot-

ing substances or their distribution in the plant may be adversely affected. In this connection it is interesting to note that the first appearing leaves are not reduced in width appreciably but are decidedly reduced in length. The second and third appearing leaves are decidedly reduced in width as well as in length. Here the reduced activity of growth substances may be accompanied by a reduction in nutrition due to smaller photosynthetic areas.

On the other hand, a saprophytic development of the fungus upon the endosperm or upon the aleurone layer, increasing in effect during the development of the first and second foliage leaves, may account initially for the reduced size of these organs. The close vascular connections between the first two foliage leaves and the scutellum has been pointed out by McCall (6). The reduced size and consequently lessened photosynthetic activity in addition to the diversion of food stores in the endosperm by the fungus results in small growth in the third, fourth, and fifth leaves. Without the intervention of some other factor this condition would continue as long as leaves were formed, transpiration would continue to be small, and final yields of dry matter would be reduced from that of the controls. Some factor must be present tending toward recovery, which under favourable conditions for growth results in increased rather than decreased yield and consumption of water.

How the inoculated plants with the first five or six leaves decidedly stunted, compared with control plants, were able to produce further leaves that were larger than corresponding leaves in the controls, in view of their reduced photosynthetic areas, constitutes an interesting problem. Maximov (5) reviewed the works of Zalenski in Russia and Yapp in England, in which the upper leaves of plants were shown to be more xeromorphic than the lower ones. This is indicated by greater total length of vascular bundles and more stomata per unit area of leaf surface, and smaller dimensions of epidermal cells. These findings were confirmed by Heuser for wheat. Maximov's colleague Alexandrov demonstrated that the intensity of transpiration increases from the lower leaves to higher leaves on the same plant. Zalenski has shown that the more xeromorphic condition of the upper leaves is due to the deflection of water from the growing tip by the fully developed and transpiring leaves. By removing the expanded leaves of *Coleus* he found that subsequently developed leaves resembled lower leaves in a looser network of veins, reduced number of stomata per unit area, and increased size of cells.

In the recovery of inoculated wheat seedlings it may be that toxin development by the fungus either ceases or the concentration of toxins is reduced below injurious concentrations owing to increasing size in the plant so that normal growth is resumed. The reduced lower leaves divert less water from the growing point and the expanding leaves consequently are less xeromorphic and larger than in the control plants. The failure of inoculated plants at 30 and 45% M.H.C. of the soil to show recovery in the same degree probably is due to the inability of the plants to supply water rapidly enough to prevent the new leaves becoming highly xeromorphic in structure.

Another possible explanation may be mentioned. Sande-Bakhuyzen (9) states, "There is evidence in the literature that the favorable effect of supplying plants with mineral salts within certain limits does not increase the photosynthesis rate, but increases leaf area, causing increased yield. This probably means a lengthening period of leaf formation, i.e., more (and larger) leaves and consequently more photosynthates." The toxic or other influences produced by the growth of *H. sativum* in the host tissues may increase the rate of intake of mineral ions into the plant, and as the effects of the deleterious substances decrease to allow recovery, the presence of large amounts of minerals may influence the sizes of subsequently formed leaves.

No significant increase was observed in the water requirements of plants in the seedling stage owing to inoculation with *H. sativum*. However, the rate of transpiration in the seedling stage per unit of transpirational area was found to be significantly greater in the inoculated plants. To reconcile these two conclusions a more intense photosynthetic activity by the smaller leaves of the inoculated seedlings may be postulated. Miller (7) considered that one of the chief causes of higher transpiration rates in plants with small leaf surface is their ability to maintain a more saturated condition of the external cell walls of the leaves than plants with larger surface.

The application of the results obtained in the greenhouse to field conditions must be made with caution. One very important difference between the experiments reported in this paper and the course of events in the fields of Western Canada is in the time of infection. The seed sown in the field generally has only a relatively small proportion of the seeds bearing the fungus, and infection from the soil increases most rapidly as soil temperatures rise above 15° C. one to two months after seeding. There is, however, evidence in the author's unpublished work that the plants showing severe lesions of the subcrown internodes were stunted as to height, and these plants show definitely reduced yields as compared to neighbouring plants (8). There appears to be an effect on the plant similar to that found in the greenhouse, but owing to the delayed infection there may not be the same tendency to recover, or the time between infection and maturation of the plants may be too short for recovery to be marked. There is also the problem of competition. In the greenhouse experiments no competition for soil moisture and nutrients occurred between the uninoculated and inoculated plants. In the field, on the other hand, diseased plants are subjected to intense competitive conditions, from slightly affected or unaffected wheat and weeds. Under Western Canadian conditions the precipitation is small and soil moistures are usually relatively low. As shown in the greenhouse, low soil moisture levels did not permit inoculated plants to recover normally. It appears, therefore, from these considerations that complete recovery of plants from an initial setback owing to infection with *H. sativum* in the field is unlikely.

Acknowledgments

The author wishes to acknowledge indebtedness to Dr. P. M. Simmonds and Mr. R. J. Ledingham, who first delved into the influence of root-rotting fungi on water relations in wheat and turned his attention to the problems involved. To Dr. J. G. Dickson, of the University of Wisconsin, goes the author's gratitude for many helpful discussions of the work during its progress. The Dominion Department of Agriculture kindly made it possible for the writer to conduct certain phases of this work at the University of Wisconsin.

References

1. BEVER, W. M. J. Agr. Research, 54 : 375-385. 1937.
2. BURKHOLDER, W. H. Ecology, 5 : 179-187. 1924.
3. GOULDEN, C. H. Methods of statistical analysis. Burgess Publishing Co., Minneapolis, Minn. 1936.
4. LINFORD, M. B. Phytopathology, 21 : 791-796. 1931.
5. MAXIMOV, N. A. The plant in relation to water. (English translation edited by R. H. Yapp). George Allen and Unwin, Ltd., London. 1929.
6. MCCALL, M. A. J. Agr. Research, 48 : 283-321. 1934.
7. MILLER, E. C. Plant physiology. McGraw-Hill Book Company, Inc., New York and London. 1931.
8. SALLANS, B. J. Progress Report of the Dominion Botanist for 1931 and 1934. pp. 24-25. Dept. Agr. Dom. Canada, Ottawa. 1935.
9. SANDE-BAKHUYZEN, H. L. VAN DE. Studies on wheat grown under constant conditions. Food Research Institute, Sanford University, California. 1937.
10. VANTERPOOL, T. C. Proceedings World's Grain Exhibition and Conference, Regina, Canada. pp. 294-298. Can. Soc. Tech. Agriculturists, Ottawa. 1933.
11. WILLIAMS, C. B. Ann. Appl. Biol. 24 : 404-414. 1937.
12. YATES, F. and COCHRAN, W. G. J. Agr. Sci. 28 : 556-580. 1938.

STUDIES ON THE INK-SPOT DISEASE OF POPLAR[1]

By Réne Pomerleau[2]

Abstract

A foliage disease of poplar caused by *Sclerotinia bifrons* (E. & E.) Whetzel has been studied during an important outbreak in the years 1935–1937. The perfect stage of this fungus has been correctly named by Whetzel. The fungus hibernates on the ground in the sclerotial condition, and during the spring apothecia are produced on sclerotia. Inoculation of the leaves is induced by ascospores ejected from apothecia. After two or three weeks of incubation, reddish areas appear on leaves; new sclerotia are formed in the lesions in June and are ready to fall about July 15. The epiphytotic development of the disease is favoured by the following set of conditions: presence of a fairly large number of sclerotia on the ground, occurrence of a dense stand of young poplars, low temperature and high humidity before and at the time of foliation. Quite a number of trees at the sapling stage are killed and others are affected by an intense defoliation, during an outbreak. These effects are noticeable only in thickets of pure population of Aspen, which represent usually the first stage of natural reforestation under Quebec conditions.

Introduction

In 1936, special climatic conditions in the province of Quebec, doubtless also in the neighbouring provinces and the adjacent part of the United States, favoured the development of a fungus affecting poplars, and consequently a fairly important outbreak resulted. The appropriate name "Ink-spot disease of poplar" was used a few years ago to designate the disease. Although this is widely spread on the North American continent, it does not appear, under normal conditions, to be very injurious to the trees. This epiphytotic started in 1935 and reached its climax in 1936, to regress markedly in 1937.

The wide occurrence of the disease enabled the writer to study its development and its effect on poplar; a certain number of interesting and unpublished facts regarding the biology and ecology of the parasite were also noted. In this article are discussed the results of a study of this quite unknown fungus. Material was collected for the histological study of the diseased leaf and the cytological investigation of the pathogen, but these will be reported in another paper.

In the following pages, an historical sketch of the disease is accompanied by notes regarding its range, importance, and the species affected; it is followed by a discussion of the identity of the fungus and a study of the biology of the pathogen, of the factors favouring its development, and, finally, by some observations on its pathological effects.

[1] *Manuscript received November 29, 1939.*
Contribution from the Laboratory of Forest Pathology, Department of Lands and Forests, Quebec.

[2] *Forest Pathologist.*

History

The ink-spot disease of poplar has been known in the United States and Canada for a good many years. As early as 1890, Ellis and Everhart applied the name *Sclerotium bifrons* to the imperfect stage of the fungus in question.

Although its occurrence had been recorded in New England, in the Canadian Rockies, and elsewhere, its perfect stage was unknown until recently. In 1920, Hartley and Hahn (6) pointed out that sclerotia had been collected in various places in the United States and Canada, but that sexual fruit bodies were not observed. Overholts (7) reported the fungus from Colorado, but he has found no sign of fruiting bodies. The first mention of the perfect stage of *S. bifrons* was made by Seaver and Shope (11). These authors state that the apothecia arising from sclerotia on the ground were found in abundance at the foot of poplars in Colorado. They state also that the name "Ink-spot fungus" was given by Dr. T. D. A. Cockerell. Later, some authors used the name *Sclerotinia bifrons* (Ellis & Everhart) Seaver & Shope to designate the organism. It will be seen later that their use of this binomial was an error.

In 1935, the writer reported (8) the presence of the fungus on different species of poplars in Quebec and finally, in 1936, it was held (9) responsible for a fairly important outbreak on aspen.

Range and Importance

The above historical sketch shows that this fungus is an American endemic species occurring almost everywhere in the United States and Canada in association with *P. tremuloides*. As this species is distributed from one ocean to the other in Canada, in the northern states and the mountain regions of western United States, it may be said that *S. bifrons* is spread throughout the whole temperate region of the North American Continent.

The phenological observations presented in this paper, coupled with those of Overholts (7), show, however, that the organism prefers the fairly cold climates. Moreover, in such states as Colorado and Wyoming, it was observed only in the mountain regions. Hartley and Hahn (6) report that in 1920, in the collection of the Department of Forest Pathology in Washington, there were 20 collections of the fungus, 15 of which were made by Dr. G. G. Hedgcock in Colorado, and five in New England and New York State by Dr. Spaulding, G. L. Barrus, and themselves. Other collections from Wisconsin, Utah, Ontario, and Virginia are also mentioned.

In Quebec, it was found almost everywhere. Previous to 1935, severely affected stands of *P. tremuloides* were rather rare. Occasionally a few Lombardy poplars growing in damp and cold places showed a fairly large number of diseased leaves. But in 1935 the writer observed a rather bad attack, especially in the vicinity of Berthierville. In the same year, a fairly severe epiphytotic was reported from the vicinity of Rimouski, where about 50% of the leaves in a stand of *P. tremuloides* were killed. However, it was in

1936 that the parasite developed in an unusual way. During that year, the presence of the disease in the epiphytotic condition was reported from the four corners of Quebec, and it was ascertained by personal visits that in certain young stands not infrequently more than 75% of the foliage was affected. In a fairly large number of cases, an almost complete defoliation had resulted from the spread of the pathogen, thus bringing about the death of young individuals.

Species Affected

The occurrence of this parasitic fungus on *P. tremuloides* and *P. nigra* has been known for many years. It appears as if the former species were the chief suscept of the pathogen. Dr. Spaulding, as reported by Hartley and Hahn, has noted "that in mixed stands of *P. tremuloides* and *P. grandidentata* only the former is attacked," in the Adirondacks. The author noted this fact also several times. In 1936, in Berthierville, there were a large number of *P. grandidentata* entirely free from the disease, whereas the trembling aspens were very badly affected. On June 22, 1937, several individuals of *P. grandidentata* were found in a fairly damp forest of Parks Reserve (Kamouraska County) with the leaves infected and bearing sclerotia of *Sclerotium bifrons*.

In 1937, the author reported (9) the discovery of the fungus on a leaf of the Carolina poplar (*P. canadensis*) in Berthierville (Plate I; 1). There appears to be no other species of poplars on which the fungus was recorded. Perhaps some unpublished data might increase the list of suscepts of the parasite.

Identity of the Pathogen

The name *Sclerotium bifrons* was used until 1930, when Seaver and Shope (11) proposed the combination *Sclerotinia bifrons* for the fructification they found on sclerotia in Colorado. This binomial has since been used at least twice in text-books to designate the fungus, for the stipitate apothecia arising from the sclerotium belong to the *Sclerotinia* type (11).

However, the question arises as to whether the organism reported by Ellis and Everhart is exactly the same as the one found by Seaver and Shope. Professor Whetzel, who made a thorough study of the species of the genus *Sclerotinia*, expressed some doubts as to the identity of the fructifications found in Colorado. Morphological studies of the perfect stage of the fungus collected at Berthierville extended over two seasons, but as a paper on this species will be published soon by Dr. Whetzel, there is no need here of a long discussion on the matter.*

It is felt that the species described and illustrated by Seaver and Shope cannot be homologated with the organism that the author found growing from sclerotia formed on poplar leaves. From a comparison of Plate I; 7, with

* *Since the present paper was written, Whetzel (12) has described the perfect stage of the true causal organism, giving the history of its discovery and the correct name. According to an article of the International Rules of Botanical Nomenclature, F. J. Seaver, in the same number, rejects the name S. bifrons Whetzel and proposes S. Whetzelii for the species on eastern poplars.*

figures given by Seaver and Shope, discrepancies can be easily detected. By colour and measurements these differences are also quite noticeable. Characters of the asci (Fig. 1) cannot fit for both organisms.

Ellis and Everhart designated this fungus under the name *Sclerotium bifrons*, after a collection made by Dearness in London, Ontario, but they gave no description. The type-collection is No. 2554 of the "North American Fungi". Saccardo (10) gives the following diagnosis: "167. *Sclerotium bifrons* Ell. et Ev. N.A.F. n. 2554 (absque diagnosi).—Stromatibus sparsis, amphigenobifrontibus, discoideis, latis, purpureo-nigris, tenuiter rugulosis, 4–6 mm. diam., intus albidoroseis, tandem excisis et foramina in foliis relinquentibus. Hab. in foliis dejectis Populii, London, Canada, 1890. (Dearness)."

This diagnosis fits well the material collected here. It appears logical, however, that this description be completed since the perfect stage is known. Publication of these notes was delayed until Professor Whetzel's description of the perfect stage, in which he gives a correct name to the organism, had appeared. To complete the present work, however, the writer's own description is given, based on the material collected in Berthierville (Quebec):

Sclerotia (4-6×2-4 mm. diam., 0.25–0.50 mm. thick) scattered on the leaves, orbicular, oblong, sometimes reniform and irregular, easily separable from the leaf at maturity and leaving a hole in falling, black brown, slightly rugose, white inside, slightly convex on the upper surface and concave on the lower one. Apothecia (4–6 mm. diam.) 1 to 3 per sclerotium, at first cupuliform, becoming almost plane at maturity, dark brown, with long stipe. Stipe up to 15 mm. in length, slender, brown, darker toward the base. Asci (130–160 × 10–13 μ) cylindrical, gradually attenuated at the base, truncate at the apex; ascospores (10–13 × 5–7μ) obliquely uniseriate, unicellular, oblong-ellipsoid, biguttulate, hyaline, with granular content; paraphysis abundant, filiform, septate, with clavate apex. (Fig. 1.)

It is not merely from the morphological standpoint that the identity of the fungus is hard to establish. Biologically also it presents some difficulties. Thus, in trying to cultivate it artificially, whether by sowing the spores or by planting a piece of the thallus on nutrient medium, the results are always negative. However, cultures develop occasionally from the ascospores of certain apothecia that form under poplar trees; these grow actively and finally produce sclerotia in abundance (Plate 1; 3). The asci have about the same shape as those of the sclerotia found on the infected leaves. In the spring of 1933, an active culture was obtained by planting debris of apparently frostbitten young poplar leaves on potato agar. In 1936, more cultures were obtained by spore discharge of fructifications on agar. On the other hand, numerous attempts were unsuccessful with certain fruit bodies well attached to the sclerotia, as were attempts to inoculate living leaves of poplar with mycelium from artificial cultures.

Careful examination of the fructifications found on the ground in the spring shows that some of them differ from the others, in that they are smaller and attached to a sclerotium embedded in the debris of leaves. In 1933,

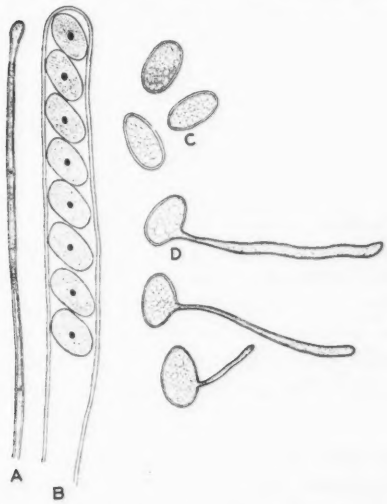


FIG. 1. *Sclerotinia bifrons (E. & E.) Whetzel. A. Paraphysis; B. Fertile portion of the mature ascus; C. Ascospores; D. Germinating ascospores (× 500).*

specimens were sent to Professor Whetzel, who stated in personal communications that, in unpublished work, he applies the name *Sclerotinia pseudobifrons* to this saprophytic species, which occurs almost always among the dead leaves of poplars affected by *Sclerotium bifrons*. The writer's observations on this form agree with those of Dr. Whetzel. The culture obtained in 1933, from dead parts of partially frozen living leaves of Lombardy poplar, should be regarded also as belonging to this species.

Another aspect of the problem of the identity of the fungus and of its developmental forms deserves mention. Davis (2) pointed out the presence of an imperfect fungus, *Myrioconium comitatum*, associated with *Sclerotium bifrons* and *Sclerotium foliicola* on willow. Davidson and Cash (1) believe that there is reason to consider it as being the imperfect stage of some species of *Sclerotinia*. As this stage was not found in the course of the present study, neither its authenticity nor its relations to the perfect stage are contested.

Biological Observations

A fairly important infection observed in a young mixed stand of aspens (*P. tremuloides*) and old field birches in the forestry nursery of Berthierville, in 1935, gave the writer the opportunity to study this disease, which was then almost unknown, and to describe the life cycle of the causal agent. During that year, a large number of leaves bearing spots and sclerotia were collected before their fall. These leaves were placed on the soil and covered with a screen to prevent their dispersion until the following spring.

The sclerotia were thus readily available while being kept under natural conditions, which enabled the writer to follow the different developmental stages of the fungus from the beginning of the season of vegetation in 1936. In that spring, however, the sclerotia of the previous year that had been disseminated naturally were numerous enough on the ground to be found without much difficulty.

Hibernation

The only organ of preservation of the parasite is, according to all evidence, the sclerotium, since it is the only portion of the organism that survives in the fall. Should the existence of an imperfect stage be established, this stage might play a role equivalent to the microspores of *Sclerotinia Gladioli* (4), but nothing is known of their capacity to serve as inoculum in the spring. It will be seen, however, that the ascospores alone suffice to bring about the infection when the environmental conditions are favourable.

Fructification

The author tried to obtain fructifications artificially on several occasions. Some sclerotia were placed in damp sand and kept at a fairly cold temperature in an electric refrigerator for a longer or a shorter period of time. When the sclerotia had not been allowed to overwinter under the snow, the results were negative. Consequently, the sclerotia collected in the summer or the autumn that follows their formation are not able to fruit. This is true also of those that passed the winter elsewhere than under the snow, for example, in the laboratory.

But sclerotia normally overwintered under the snow behaved differently: when placed in the refrigerator at a temperature slightly above the freezing point, they fruited abundantly very early in the spring, although the development of the fructifications on the margin of the sclerotia ceased as soon as they were brought to room temperature (Plate I; 10); when kept at room temperature on moist sand, they remained sterile.

From these laboratory experiments it appears that this fungus is able to form its sexual organs of reproduction only at relatively low temperatures and after overwintering of the sclerotia on the soil.

Outdoors, the fructifications are formed rather early in the spring, that is, a short time after the melting of the snow. Beginnings of ascophores were found as early as April 24, in 1936, but the development of the asci had not yet taken place. These organs, however, developed only on very damp soil and when protected from the sun by fairly dense shrubbery. Shortly thereafter, and coincident with the opening of the aspen buds, a large number of apothecia had formed and were ready to discharge their spores (Plate I; 6). This active phase of the parasite coincided with the foliation of the poplars, and occurred in Berthierville about May 7, in 1936. The fructifications developed much later in 1937. The first signs of their development appeared only about May 10, and the spores did not reach maturity before May 29,

though all the poplar leaves were expanded and had reached their full size some time previously.

Dissemination

As the fructifications of the fungus are formed on the soil, the spores must be discharged violently in order to bring about infections on the leaves, which are the only easily affected organs of the suscept. This ejection was brought about artificially by placing an apothecium rapidly in water without, however, covering the fertile surface. If the fruit bodies are matured, the spores then are discharged into the air with a light explosion that produces a cloudiness visible at a short distance from the ascigerous surface. According to this observation, it seems reasonable to believe that, in nature, the ascospores are ejected into the air when water moistens the fructifications during a period of rain. They are disseminated by the wind which brings them in contact with the leaves, according to the hydro-anemophile type of dissemination.

Germination

The ascophores collected and examined at the beginning of May, 1937, contained a large number of asci with well formed spores. These spores, when placed in a drop of water, germinated abundantly, and germ tubes 60 to 80 μ in length were observed after 20 hr. (Fig. 1). Although germination occurs easily in water, nevertheless the development of the mycelium does not exceed a certain stage on a substratum other than the poplar leaves. Thus, the spores discharged on potato agar germinated as well as in a drop of water, but the growth of the germ tubes soon came to a standstill.

Consequently, in nature the spores should germinate rapidly once transported to the poplar leaves, since the substratum is moistened with rain that brought about their ejection.

Inoculation

It was very easy to reproduce this part of the life cycle artificially in the laboratory. For successful inoculations it sufficed to atomize a suspension of spores on the surface of the leaves, and to maintain for 24 hr. the humidity of the latter by means of celluloid chambers built for this purpose (Plate I; 9). A few young potted trees, inoculated in this manner in the laboratory on May 13, showed on June 4 very conspicuous and characteristic spots on some of the leaves.

Infection

In 1936, the symptoms of the disease appeared on outdoor trees about June 5, a month after the opening of the buds. The pathological signs were sufficiently characteristic at that time not to be confused with those of other infections (Plate I; 2). They are, at the beginning, more or less extensive spots, which are separated clearly from the rest of the leaf tissue by reddish or dark brown discolorations. Not infrequently the whole leaf is thus discoloured. This change in the appearance of the affected leaves usually takes place very rapidly; in one or two nights the foliage of the trees may thus be

discoloured to an extent varying with the intensity of infection (Plate I; 2, 8). Usually the spots are circular or irregular; however, it frequently happens that the infected areas are narrow and linear (Plate I; 5). This seems to result from the fact that the mycelium develops only in those parts of the leaf that are abundantly moistened, as along a streak of water. Moisture is therefore a factor of primary importance upon which depends the severity of the infection. On the whole, the droplets or a film of water must remain on the leaves long enough to allow the penetration of the mycelium.

Most of the infected leaves showed symptoms of the disease between June 1 and 8. Practically no subsequent spreading of the lesions that had invaded the leaf blade at that time occurred. Thus, the growth of the mycelium in the leaf tissue is very rapid. This conclusion is substantiated by results from the inoculations carried on in the laboratory. It seems also that the extent of the infected area is dependent upon the degree of moisture available during the incubation period. Small foliated branches kept continually, from the time of inoculation until the appearance of symptoms, in a water-saturated atmosphere showed much larger spots than leaves left in the open air immediately after inoculation, that is, two or three days after the atomizing of the spores.

The temperature factor, aside from conditioning the development of the fructifications, seems to influence also the growth of the mycelium in the leaf. Thus, it was found that infections developed in the laboratory and at a higher temperature were not as severe as those developed in the open; both were inoculated at the same time and in the same manner. Also the development of the disease in the open is inhibited by the rise in temperature with advance of the season. This fungus is, therefore, a parasite inhabiting cold regions and developing early in the spring.

Formation of the Sclerotia

The infection period, including incubation and appearance of the lesions, was practically completed by June 15, 1936; as the temperature of the air became fairly warm, all growth ceased, except for the formation of the organs of preservation, the sclerotia, which marks the end of the vegetative activity for the year. The sclerotia showed on June 15 the first evidences of their formation. Felt and Rankin (5) state that the sclerotia of this fungus develop on the dead leaves in the fall. This is obviously incorrect because, on July 15, in Berthierville, the sclerotia are not only formed, but very often completely mature and lying on the ground.

At the beginning of this stage, one or several pale, circular or oblong spots may be seen in the infected portion of the leaf. Examined with transmitted light, these regions are paler than the rest of the leaf. They are the very places where the sclerotia arise somewhat later. These small circular spots appear almost always after a few days of rain; but once started, the transformation of the mycelium in a given area of the leaf is very rapid. In fact, usually a few days suffice for the translucent spot to become thicker than the surrounding portion of the leaf and to assume at first a brownish-red coloration,

which turns almost black at maturation (Plate I; 4). Shortly after the appearance of these spots, the remaining portion of the leaf becomes slightly distorted and assumes a concave-convex appearance at the margin of the developing sclerotia.

The formation of the resting organs in the infected portion of the leaf may occur rapidly if the diseased leaves are placed in vessels with moistened paper in the bottom. The abundant degree of moisture that conditions largely all the other developmental stages, determines also the formation of the sclerotia. These organs develop with difficulty or not at all on the infected leaves of a potted plant kept in a rather dry atmosphere in the laboratory. On the other hand, if the foliage is covered with a bell jar after an abundant watering, the sclerotia usually appear without delay.

The number and the size of the sclerotia vary considerably. It seems that this variation might be ascribed, to a large extent, to the size of the infected portion of the leaf. The following experiment was made to clarify this point. Leaves collected outdoors on June 1, 1936, were placed in moist chambers. Examined each day, these leaves, 35 in number, had produced only two small sclerotia on June 9. This first lot of leaves, with only small infections, hardly produced any sclerotia, probably because the mycelium, which was only slightly developed, had ceased to grow even in the presence of moisture, on account of the room temperature being too high. Another lot of leaves, collected on June 5, gave different results, which are summarized in Table I.

TABLE I

RATE OF FORMATION OF SCLEROTIA ON INFECTED LEAVES

	June 5	June 7	June 8	June 9	June 10
Leaves without sclerotia	90	68	41	24	22
Leaves with sclerotia	0	22	49	66	68

It may be noted in the above table, that 68 out of 90 leaves had produced sclerotia after five days of observation, and 22 failed to do so. This shows that the mycelia in the leaves collected June 5 had developed sufficiently since June 1 to be able to produce a large number of sclerotia even at room temperature. In order to complete the study of the influence of the size of the lesions on the development of the sclerotia, more leaves were submitted to the moist chamber experiment, but this time they were divided into three groups of 25 according to the percentage of infected surface.

Table II, which summarizes the results of this experiment, shows that several sclerotia developed in badly diseased leaves, whereas almost every one of those showing only a portion of discoloured surface produced very few or none. Sclerotia were obtained also from the leaves artificially inoculated in the laboratory, but this occurred only in the case of fairly severe infection.

TABLE II

DEVELOPMENT OF SCLEROTIA ON LEAVES INFECTED TO VARIOUS DEGREES

Percentage of infected surfaces	Number of leaves without sclerotia		
	June 8	June 9	June 10
100	25	5	1
50	25	22	18
25	25	25	25

The development of these organs does not appear to be affected by the drying of the surface of the leaves for a few days after the discoloration of the tissue, provided, however, that the surface be abundantly moistened again.

Fall of the Sclerotia

The fall of the sclerotia usually begins shortly after their formation and maturation. This happened about June 15, in 1936. Usually a fissure appears in the leaf tissue at the margin of the sclerotia, the latter separate and fall, leaving holes in the blade which have exactly their shape; but occasionally the whole leaf falls before the resting organs. At the end of July it is already fairly difficult to find sclerotia on the trees, or if present, the mere touching of the leaves is sufficient to provoke their fall to the ground. In colder regions of Quebec, however, the sclerotia develop and remain on the leaves later in the season. On the ground, the sclerotia await the favourable conditions of the following spring to resume the cycle.

Considerations on the Life Cycle

The life history of this pathogen may be summarized as follows:

1. Hibernation on the ground in the sclerotial condition.
2. Development of apothecia on the sclerotia at the beginning of spring.
3. Ejection and dissemination of the ascospores shortly thereafter.
4. Incubation period varying from 15 days to 3 weeks after inoculation of poplar leaves.
5. Appearance of spots toward the end of spring.
6. Development of sclerotia on poplar leaves at the beginning of summer.
7. Maturation and fall of the sclerotia 15 days or a month later.
8. Resting period extending until the following spring.

In a diagram (Fig. 2), this life cycle is represented in the order of succession of the different stages, and is superimposed on the period of vegetation of the suscept. The diagram is based upon the behaviour of the pathogen and its suscept in 1936, when the epiphytotic was most severe. In other years, perhaps the stages of the cycle will not appear exactly at the same dates and will not necessarily correspond with the same stages of the development of the poplar foliage. As the biological observations of 1936 were made when

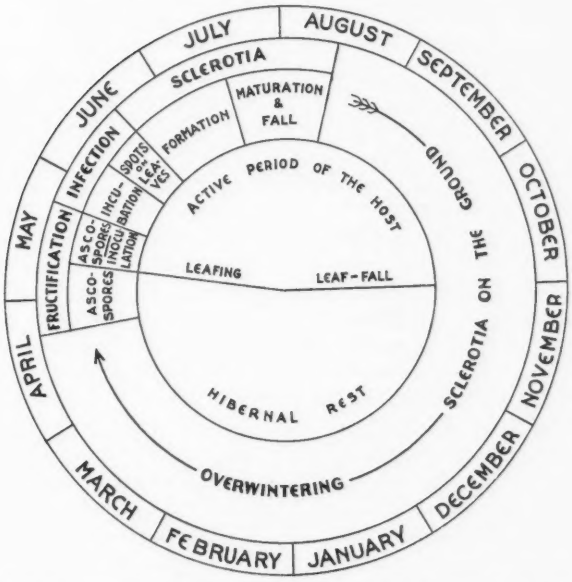


FIG. 2. *Diagram representing the life cycle of Sclerotinia bifrons and the main phases of development of leaves of Populus tremuloides.*

the conditions of infection were ideal, it is considered that the important stages in the development of the parasite and the parasitized coincided perfectly.

On the whole, this organism has a very short life cycle, which includes a single generation of spores to enable the dissemination of the disease. The fruiting period must therefore begin before the foliation of the suscept in order to provide the necessary inoculum for the period of receptivity of the latter, that is, when the young leaves are still sensitive to the infection. If the climatic conditions are favourable at that time, the stages of development will follow each other in the order mentioned above, and will end with the fall of the resting organs and a more or less severe defoliation of the affected trees.

Ecological Observations

Periodically, the disease seems to appear more severely in certain regions. In no place, however, have the reasons of this recrudescence in the strength of the parasitism of the fungus been determined. The epiphytotic of the ink-spot disease of poplar in 1936 enabled the writer to point out the influence of the climatic conditions on the development of the causal agent (Fig. 3).

The present phenological observations, though incomplete and restricted to only one season and to a single locality, have enabled the writer, however, to outline the conditions that may bring about the epiphytotic.

The stages in the life cycle of the parasite, which were recorded in the order of their appearance in 1936, were correlated with the temperature and humidity for the period of infection. In Fig. 3, curves showing weekly means of temperature and air humidity at the Berthierville nursery for the years 1934 to 1937 appear to be fairly significant. In considering the behaviour of each one of these factors during the season of vegetation of 1936, it is noted first that the temperature remained fairly low, with an average of less than 35° F. until the first few days of May; and second that the moisture of the air during the first two weeks of May was relatively high, with an average of about 90%. From this it appears that the low temperature in April and the beginning of May favoured the development of the fruit bodies of the parasite, and that the high degree of moisture and the abundant rains facilitated the inoculation of the suscept, which was just in process of foliation in May.

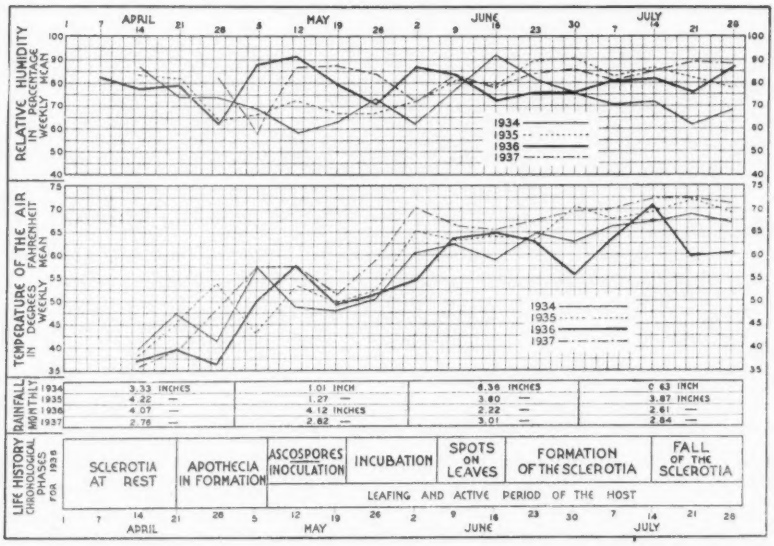


FIG. 3. *Graphic summary of the main phases of the development of the disease in relation to certain climatic factors during the years 1935, 1936, 1937.*

With such a good beginning, the disease could hardly fail to reach an epiphytotic rate. Once started in the leaves, the infection could hardly fail to proceed, especially if favoured by the recurrence in June, as happened in 1936, of damp and relatively cold periods that coincided with appearance of the spots and development of the sclerotia.

A comparison of the curves of the climatic factors for the two years that preceded the epiphytotic with those of the same factors but for the year that followed it shows that it is possible to explain, at least partially, the failure of the disease during these three seasons. It is noted that the temperature rose

much sooner at the beginning of the seasons 1934, 1935, and 1937 than in 1936. Furthermore, and this is perhaps the most important point, the degree of moisture was very low during the period of infection, that is from April 15 to about May 15, thus inhibiting the development of the fruiting organs and consequently the dissemination. If, later, however, a moist atmosphere lasts a few more days, the temperature may then be too high, or the substratum no longer in a state of receptivity, as the cuticle of the leaves may have hardened.

In 1934, the attacks of the fungus on the foliage were very weak. The following year, a notable proportion of the leaves, especially in shaded places on the border of the wood, was infected. An epiphytotic followed in 1936 to decrease to almost nothing in the spring of 1937. The extent of the disease was very severe in 1936, mostly on account of eminently favourable conditions, but the abundance of the inoculum had also something to do with it. In fact, the infection of 1935, though fairly weak, did nevertheless contribute to a general infestation and supplied the inoculum for the following year. The writer is convinced that the climatic factors control largely the development of the parasite, but the occurrence of a fairly important centre of infection in a stand is also a factor that should not be neglected.

Another condition essential to the occurrence of an outbreak is a fairly dense stand of young poplars with the foliage not too far from the soil. Although without accurate measurements on the influence of the density, purity, or age of a population upon the degree of infection, the disease was observed to be especially severe in places where a large number of young poplars were growing. It is well known that this type of stand occurs frequently in the Province of Quebec in natural reforestation a few years after the disappearance of the first stand by cutting or fire, or the abandonment of cultivated lands. The young birches or aspens then form a thicket during the period preceding the large sapling stage.

The author's observations were made chiefly in a stand that consisted almost exclusively of poplars mixed now and then with birches. Consequently, with the climatic conditions which prevailed in 1936 and abundant inoculum on the ground, the outbreak could not fail to be severe at the border of an old wood. This was as serious, if not more so, as any in all the regions that were visited in the Province of Quebec and also in numerous places reported to the writer. The pure young stands, however, were those especially affected.

The set of conditions essential to an epiphytotic development of the parasite appears to be that mentioned above, for the disease remains mild or disappears if a definite factor fails in the environment. Such was the case in 1937. In the same plot that was badly affected the previous year, very few leaves were infected, in spite of the abundant inoculum on the ground. This decrease may be easily accounted for by the rapid rise in temperature in the middle of April, followed by a dry period.

It could be objected to this explanation that the failure of fruiting of the very abundant sclerotia resulted from the unfavourable conditions of the winter or from any other unknown cause. It could be answered that these

organs of preservation had all the necessary vitality, for they produced fruit bodies in a refrigerator and even outdoors on a protected cold soil.

On the whole, the conditions favouring the epiphytotic development of the disease are the following: (i) Presence of a sufficient inoculum: sclerotia in fairly large numbers on the soil; (ii) Occurrence of a dense stand of young poplars; (iii) Fairly low temperature before or at the time of foliation; (iv) Fairly high degree of moisture during foliation.

Pathological Observations

In the course of this study, observations were made as to the effects of the disease on the suscept. Material was collected and prepared for histological study of the diseased tissues of the leaf, but this still fragmentary work will not be considered here. The following considerations deal rather with the effects of the infection on the tree as a whole and therefore on the forest population.

In 1935, the limits of a sample plot were marked in the young stand which served for making almost all the above observations. All the trees in the plot were numbered and an enumeration, for each tree, of healthy and diseased leaves was effected before the fall of the latter. Taking into account the dead trees also, this count was repeated in 1936 on the same individuals. The results are summarized in Table III.

TABLE III

NUMBERS OF LEAVES INFECTED BY *Sclerotinia bifrons* IN A SAMPLE PLOT OF *Populus tremuloides*

	1935			1936		
	Total number	Average per tree	%	Total number	Average per tree	%
Trees	74			74 (11 dead)		
Leaves	31,186	421.4		21,338	288.3	
Healthy	30,195	408.1	96.9	2,172	29.3	10.2
Diseased	991	13.3	3.1	19,166	259.0	89.8

It may be seen at once in the above table that the infection was considerably more severe in 1936 than in 1935. In spite of the small percentage of disease in 1935, 11 trees died as a result of a total defoliation or a partial reduction in the leaf surface. This infection had additional effects in reducing considerably the vitality of the trees that remained alive. So instead of an increase in the number of leaves in 1936, as should normally occur after a year of growth, a decrease was recorded.

Unfortunately these counts were not repeated in 1937 to show the effects of the previous infection, which was so much more severe than in 1935. How-

ever, a superficial inspection of the trees showed that a large number of them were dead, the foliage was pale, and the shoots were short and few. The mortality was high especially among the young trees. The taller individuals, from 8 to 12 ft., though severely affected, were not killed because a fairly large proportion of the uppermost leaves remained healthy in 1935.

On the whole, the small trees may be killed by a severe infection in the course of an outbreak, but the taller individuals are rarely affected to such an extent as to die. From the standpoint of the tree population, the damage cannot be very serious even in the case of a widespread epiphytotic, for the smaller trees are frequently doomed to disappear sooner or later anyhow, being suppressed by the stronger and taller individuals, which recover with the return of normal conditions. However, in certain stands or plantations to be used for special purposes, such as wood for matches, the losses may be fairly heavy.

Conclusions

The results of investigations and observations on a disease that appears to be important in certain years are presented as a contribution to the study of the causal fungus under all its aspects, practical as well as scientific.

This organism is, according to all evidence, a North American endemic, with a geographical range that appears to coincide somewhat with that of *Populus tremuloides*, its chief suscept. It was found also on other American species of the same genus, and on the Lombardy poplar, but always within the range of the trembling aspen.

The identity of the parasite is now well established and its perfect stage has recently been properly named. Whetzel was the first to find the fructification of this fungus and to separate it from certain associated saprophytic species.

The following conclusion should be drawn from the results of the biological and ecological observations presented above: this fungus, which produces annually a single generation of spores and requires fairly favourable conditions for its development, cannot be considered a very important destructive agent of trees and tree populations. It usually happens that one or other of the conditions essential to infection fails to appear simultaneously with the others and at the proper moment. It is possible, however, that young stands of poplars, which have a greater value than the thicket representing the first step of reforestation, may be more severely affected by this disease, either because the environment might be very favourable to the development of the pathogen, or because susceptible species, strains, or varieties might have been introduced or developed.

As a final consideration, it may be added that this American indigenous pathogen has rather mild effects under normal conditions. It might be quite different with a parasite of the same group but of foreign origin, because this might assume the highest degree of virulence on one or several of our forest species, even under the usual conditions.

Acknowledgments

The writer wishes to express his appreciation to Dr. H. H. Whetzel, Professor of Plant Pathology at Cornell University, for his valuable suggestions and criticisms in revising the manuscript. The writer is also indebted to Mr. Emile Jacques, graduate student at Cornell University, who has translated this paper originally written in French.

References

1. DAVIDSON, R. W. and CASH, E. K. Mycologia, 25 : 266-273. 1933.
2. DAVIS, J. J. Trans. Wisconsin Acad. Sci. 19 : 686. 1919.
3. DAVIS, J. J. Trans. Wisconsin Acad. Sci. 20 : 414. 1922.
4. DRAYTON, F. L. Mycologia, 37 : 46-72. 1934.
5. FELT, E. P. and RANKIN, W. H. Insects and diseases of ornamental trees and shrubs. Macmillan Company, New York. 1932.
6. HARTLEY, C. and HAHN, G. G. Phytopathology, 10 : 141-147. 1920.
7. OVERHOLTS, L. O. Mycologia, 21 : 274-287. 1929.
8. POMERLEAU, R. Annales de l'ACFAS, 1 : 71. 1935.
9. POMERLEAU, R. Annales de l'ACFAS, 3 : 92. 1937.
10. SACCARDO, P. Sylloge Fungorum, 14 : 1169. 1899.
11. SEAVER, F. J. and SHOPE, P. E. Mycologia, 22 : 1-8. 1930.
12. WHETZEL, H. H. Mycologia, 32 : 124-127. 1940.

PLATE I

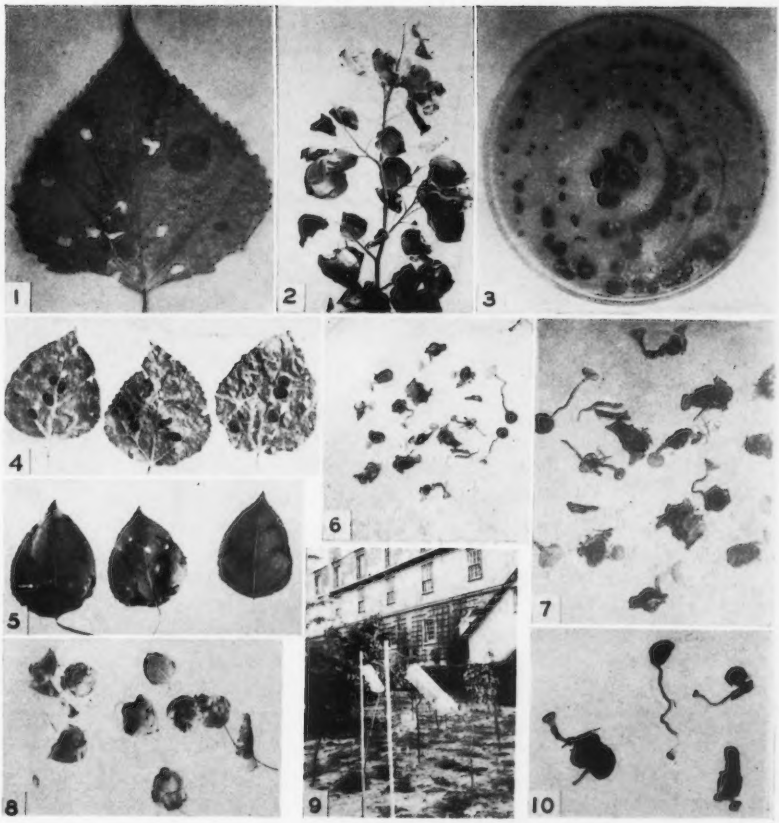


PLATE I. 1. *Leaf of Populus canadensis infected with Sclerotinia bifrons, showing sclerotia and holes left by the fall of other sclerotia* (×½). 2. *Twig of P. tremuloides with leaves all affected by the disease* (× ⅛). 3. *Sclerotia of S. pseudo-bifrons produced abundantly on potato-agar in Petri dish* (× ½). 4. *Dead leaves just before the fall of sclerotia* (× ½). 5. *Infected leaves, showing linear spots* (× ½). 6. *Sclerotia bearing one to three apothecia* (× ⅓). 7. *Matured apothecia of S. bifrons* (× ⅔). 8. *First signs of the disease on leaves of P. tremuloides* (× ⅛). 9. *Celluloid inoculation chambers used on outside trees.* 10. *Different stages of development of apothecia* (× 1).

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 18, SEC. D. MAY, 1940 NUMBER 5

ON THE LIFE CYCLE AND OTHER ASPECTS OF THE SNAIL, *CAMPELOMA*, IN THE SPEED RIVER[1]

BY J. C. MEDCOF[2]

Abstract

Judged from a statistical study, the life span of the snail is five years. Parturition occurs in summer and year classes are recognizable. Sizes of approximately 12, 17, and 23 mm. are reached in one, two, and three years respectively. Sexual maturity is reached in two years and reproduction is parthenogenetic. Some snails hibernate and some aestivate. Rest marks on shells and opercula and a limy deposit in the protoconch were studied.

In 1934 the writer's attention was directed by Mr. J. G. Oughton of the Royal Ontario Museum of Zoology, Toronto, to a colony of a single kind of freshwater snail, *Campeloma*, living in the Speed River at Hespeler, Ontario. The specific identification of the form is undecided because no careful taxonomic study of the genus has been made yet. Mr. F. C. Baker of Urbana, Illinois, has examined collections and states that the form is allied to *C. decisum* but that it is probably a new species.

Successive random samples of the snails were collected from the river on July 9, 1934, November 11, 1934, February 3, 1935, and March 8, 1936, and the shell heights measured to the nearest millimetre with calipers. A separate record was kept of the measurements of empty shells occurring in the samples except in 1935, when they were not made. The results of the measurements are presented as size-frequency distribution curves, plotted on a percentage basis, in Fig. 1. Only two curves have been prepared from the measurements of the empty shells—one for the two 1934 samples combined and the other for the 1936 measurements alone.

In their study of *C. rufum* in the Salt Fork River, Illinois, Van Cleave and Altringer (7) have justified the treatment of clear modal groups such as appear in the present curves as year classes. The present data are considered in the same way and summarized in Table I. Three maxima are usually apparent in each curve.

GROWTH RATE

Table I was used as a basis for constructing the growth curve, Fig. 2, by merging the data for the separate year classes represented. There seems to have been little variation in the growing conditions during 1934 and 1935,

[1] *Manuscript received January 23, 1940.*
Published with the permission of the University of Western Ontario.

[2] *Demonstrator in Zoology, 1934–1936, at the University of Western Ontario; now Scientific Assistant in Biology to the Fisheries Research Board of Canada.*

so this procedure seems justified. Although the largest reported Salt Fork River shells measured 40 mm. and were considered three years old, the writer has found Speed River shells as large as 47 mm. It seems improbable from the growth curve that this size could be attained in the Speed River in less

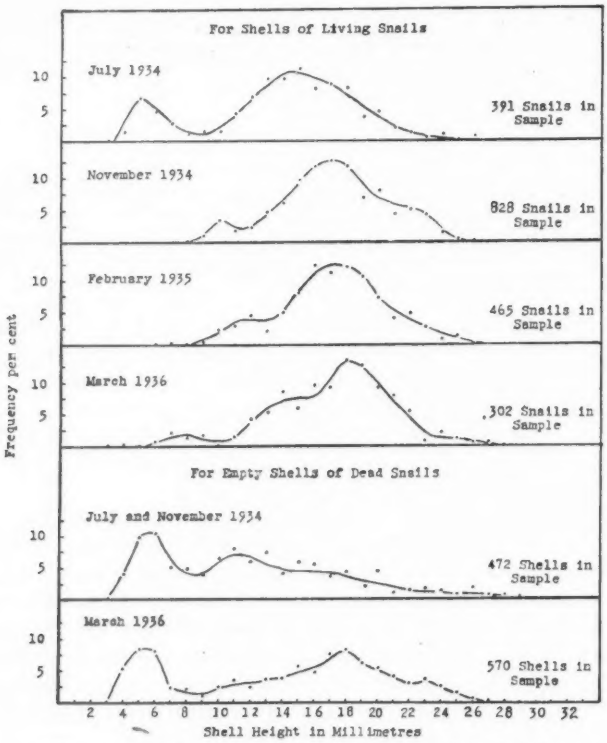


FIG. 1. *Size-frequency distribution of heights of snail shells.*

TABLE I

SHOWING MODAL VALUES TAKEN FROM FIG. 1

Date of collection	First maximum, mm.	Second maximum, mm.	Third maximum, mm.	Fourth maximum, mm.
July 1934	6	15	19	—
Nov. 1934	10	17	23	—
Feb. 1935	12	17	—	—
Mar. 1936	13	17	—	—
Empty Shells				
1934 collections	6	12	18	—
1936 collection	5	11	18	23

than five years. The northern form, then, grows more slowly, lives longer, and reaches a greater ultimate size than the Salt Fork River snail.

Winter Conditions

Fig. 2 shows that in the Speed River colony there is no slackening in the growth rate during the first winter, but that in the second there is a marked depression. The reason for this pause may be that at this time the two-year-old snails are developing their first brood of uterine young. Reference to the frequency curves for empty shells (Fig. 1) shows evidence suggesting that the winters are particularly hard on the snails. A high mortality among the newly born is indicated by the maxima at 5 and 6 mm. The writer has not attributed this to any particular cause. The other three modal values, however, at 11, 18, and 23 mm., if marked on the mean growth curve (Fig. 2) will each be found to have a corresponding "winter" position on the abscissal time scale. Winter killing is probably largely responsible for the multimodal nature of the frequency curves for empty shells.

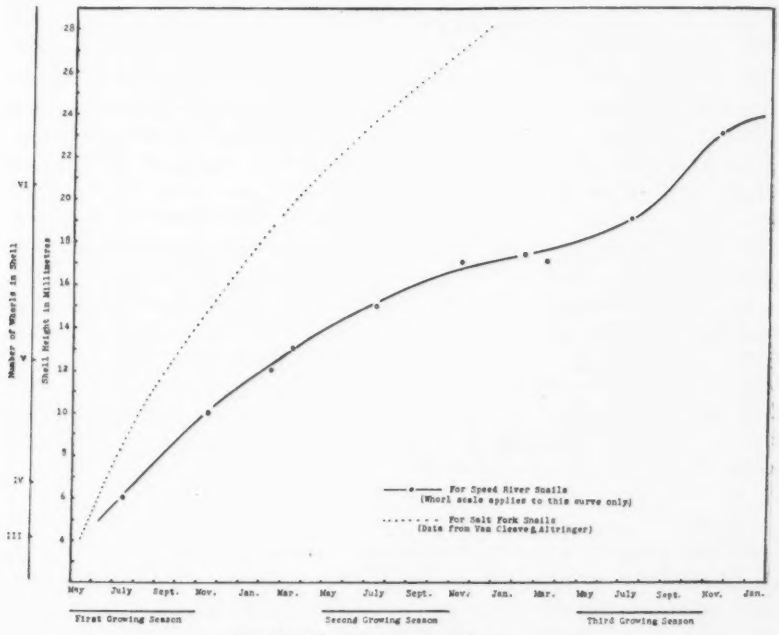


FIG. 2. *Growth rate of shells.*

Call (2) has said that in the fall of the year the snails *(Campeloma)* bury themselves deeply in the mud and hibernate until spring. Van Cleave and Altringer found that the Salt Fork River stock remained active all winter long. This apparent disagreement illustrates the flexibility of the animal's

behaviour, because the present study corroborates both observations. In the stream it was found that throughout the winter the snails continued to crawl about over the hard bottom and could be collected easily by clearing away the ice. On the same days *Campeloma* in the pond were found to be buried to some depth in the bottom, as will be described.

Above the point in the river where the sampling was done there is a mill pond, a hundred acres or more in extent, held back by a dam. The pond bottom here is of soft black muck often smelling strongly of hydrogen sulphide. Throughout the warmer parts of the year there is a congregation of snails (*Campeloma*) on the bottom beginning at the pond margin and extending out to a depth of 10 or 12 in. Here the snails are usually so plentiful that it is seldom more than an hour's work to collect 200 or 300 of them. (The reason for this summer congregation is obscure. It may be related to oxygen depletion of the water at greater depths owing to decomposition of the bottom muck.) Strangely enough, by November 10, 1935, when the surface water temperature was 6.8° C., these snails had completely disappeared from the margin of the pond but were found at greater depths by dredging from a boat. During the winter they were secured by dredging mud through a hole in the ice. After some exposure to air a number of the latter extruded their young.

On March 24, 1935, the writer was fortunate to be at the pond during the period of emergence from hibernation. The surface water temperature was 6.6° C., the ice had "gone out" on March 17, and March 22, 23, and 24 were all bright warm days. On this day there were very few snails to be found about the pond margin, but several were collected from 2 or 3 ft. of water. Many of these were crawling towards shore. In each case the pubescent covering of the shell and the operculum were impregnated with an adhesive layer of fine silt. Besides this the periostracum and even the pearly layers of the shell itself were dyed by the black muck. This dark colour persisted even after thorough washing and scrubbing and after months of preservation. None of these peculiarities are to be found in shells taken from the pond at other times of the year and never in those taken from the river itself. The shells must be bleached out to their normal greenish colour during the spring, for by July they resemble the river shells in every detail. It seems reasonable to attribute the change to hibernation burial in the muck of the pond bottom.

Aestivation

Aquatic pulmonates are known to practise aestivation and to form epiphragms in the mouths of their shells as do their terrestrial relatives (3). Aestivation is much less common among the larger branchiate types. Mr. William LeRay of the University of Toronto recounts that in August 1927 he found *Campeloma* in a vigorous condition along with crayfish under stones in the dry bed of a brook that normally flows into the Crane River near Johnston's Harbour, Ont. It is unusual to find *Campeloma* except in permanent waters, but it would seem that it can survive in temporary brooks for some time.

Reaction to Change in Water Level

An example of striking contrast in behaviour of the snails in different habitats may be cited. Rapid changes in the water level of the Speed River pond are frequently brought about by the varying needs of the mill and by other factors. These changes affect the pond snails during the summer, for they were observed to travel back and forth maintaining a position just at the water's edge. In some cases they were observed to travel as much as 12 ft. overnight in places where the beach sloped gently.

The opposite extreme was found among snails living on the flat surfaces of rocks below the dam. Here the stones were completely overgrown with a dense mat of *Cladophora* except for small circular patches which were covered by the extended feet of snails. The shells of these animals themselves bore a heavy growth of *Cladophora* and it appeared that they remained permanently attached to the small bare patches of rock. On one occasion marked animals were observed over a period of two days throughout which they did not leave their positions.

Parthenogenesis

No male of the species was found during this study, although the genitalia of approximately 450 snails were examined. Mr. J. G. Oughton has made a similar report on the results of his own earlier study of the Speed River snails. Thus it appears that parthenogenesis is the normal form of reproduction in the Speed River colony as in the Salt Fork River stock (5, 7).

Sexual Maturity and Reproductive Capacity

The smallest pregnant snail found during the investigation measured 17.5 mm. in height, contained one embryo, and was collected in February 1935. From Fig. 2 it would appear that, at the earliest, reproduction begins either during the latter part of the second summer or during the second winter. Probably in most cases it is delayed until the third summer, when the length is 19 mm. It is at about this same size or slightly larger that the Salt Fork River snails begin to reproduce, but they are then only one year old. The northern form seems to have a delayed sexual maturation.

TABLE II

The relation between size, age, and reproductive activity of 55 snails collected in February and March 1935

Age of snail, years	Size of snail, mm.	No. of this class pregnant, %	Average number of eggs and/or embryos in pregnant uteri
1	5 – 12	0	0
2	13 – 18	18 (high)	2
3	19 – 23	77	6
4	24 – 28	93	15

Table II summarizes the results of uterine examinations made on snails collected early in the spring before parturition began. There is a clear, directly proportional relation between the size of the parent and her reproductive capacity. The highest fertility record, 22 mature and 4 developing embryos, was that of a 28 mm. snail taken from the mill pond on March 24, 1935. A study of Table II with reference to Fig. 1 will demonstrate that the maintenance of the colony depends almost wholly on the reproductive activity of the three-year-olds. The stock of *C. rufum* in Illinois, as shown by Van Cleave and Altringer, is more prolific and probably maintains itself chiefly through reproduction by the two-year-olds.

Table III shows the results of uterine examinations made on snails collected at different times of the year. From this it seems that ovulation and embryonic development go on at all seasons but that parturition is intermittent and occurs only in the summer. In other words, the mature embryos accumulate in the uterus during the winter. These conclusions are much the same as those of Van Cleave and Altringer.

TABLE III

THE RESULTS OF UTERINE EXAMINATIONS OF 73 SNAILS 20 MM. OR MORE IN HEIGHT

Date of collection	Number examined	Empty, %	With eggs or very small embryos, %	With large embryos, %
July 1934	21	33	48	24
Nov. 1934	15	20	60	67
Feb. and Mar. 1934	37	11	78	89

REST PERIODS IN SHELL DEPOSITION

Careful measurements of the height, width, and number of whorls of shells and the heights and widths of the corresponding opercula have shown that there is a precise relation among all these. It has therefore been possible to supply two size scales to the growth curve (Fig. 2), one showing the shell height and the second showing the number of whorls.

Practically all snails more than one year old show at least one heavy brown line paralleling the growth lines somewhere on their shells. The opercula too, ordinarily show one or more concentric rings that stand out more clearly than the growth rings. A study involving the measurements of the relative positions of these heavier lines on the shells and opercula has convinced the writer that the two are formed simultaneously.

The peculiar conditions that favour the formation of these lines have not been discovered. However, a number of specimens that failed to grow appreciably while in a laboratory aquarium for eleven months invariably showed a broad dark brown band at the edge of the shell. It seems likely that line-formation is associated in nature with rest periods in shell deposition, but the occasion for these periods is still in doubt.

Shell Erosion and Structure of the Spire Tip

A large shell dropped into weak acid begins "gassing" at the protoconch immediately but only there, showing that in this oldest region of the shell the limy part is least protected by the periostracum. Shell erosion in the Speed River colony was conspicuously absent, probably because the stream flows through country where the main outcrop is limestone. In some habitats, on the contrary, several of the uppermost whorls may be completely missing from old shells, which may be scarred additionally with deep furrows or pits scattered over other parts.

The writer was curious to know if this removal of the upper whorls inconvenienced the snail and how repairs were made to the shell when it was perforated by erosion. To study this, median vertical sections were made of several shells. It was discovered that although the newly-born snail occupied all the whorls of its shell, it very soon began to fill in the smallest ones with a solid limy deposit. This process appeared to be continuous, a total of 0.7, 1.7, 2.4, and 3.0 whorls being completely filled in by the time the snail reached 10, 20, 30, and 40 mm. in height respectively. The fact that the snail normally abandons the cavity of the protoconch suggests that erosion of the solid part of the spire would not interfere seriously with the life processes.

A microscopic study of thin sections of the shell showed this central secretion to be structurally distinct from the three typical layers. The new deposit is probably secreted by that part of the mantle covering the visceral hump, while the periostracum, ostracum, and hypostracum seem to be formed at the mantle margin (6). With this probable difference in origin it might be expected that the structure would be different. It seems likely that a secretion of a similar type would be useful in making repairs to the shell when it is perforated by erosion.

Reversal of Shell Symmetry

Reversed shells are not infrequently met with in this genus but in the Speed River stock they are rare. Of 530 uterine embryos examined 2 were sinistral, and in a sample of 870 snails above the age of one year, only 2 were sinistral. This is low compared with the Salt Fork River variety (4).

Enemies of *Campeloma*

Baker (1) records several fish, amphibian, reptilian, and bird enemies of *Campeloma*. The writer has no direct evidence of fish preying upon the snail in the Speed River, but he has examined only five stomachs, from two common suckers, two bullheads, and one dace, all large enough to take snails. The writer examined the stomachs of nine bullfrogs *(Rana catesbiana)* from the pond and found that while tadpoles and small frogs constituted a greater part of the diet, snails *(Campeloma)* of various sizes formed an estimated 24% of the bulk of the stomach contents.

Only one observation was made on birds as possible enemies and this was not in the Speed River but near Burnt River at Coboconk, Ont., June 9, 1934.

Freshly regurgitated pellets of the European starling were found at the base of a dead pine tree where the birds were nesting in what had been the nest of a flicker. The pellets contained three snails *(Campeloma)* measuring 20, 22, and 26 mm. in height. None of these had been digested, for the bodies and opercula were still intact within the shells.

A Mite in the Mantle Cavity

Living in the mantle cavity of the snail was a mite, kindly identified by Miss Ruth Marshall as *Unionicola campelomaicola*. In the river proper, 18% of the snails were infested but rarely was there more than one mite in a snail. In the pond stock there was a 46% infestation with an average incidence of 1.6 mites per infested snail.

Besides the above, an insect, *Mesovelia bisignata* (Uhler), identified by Dr. J. D. Detwiler of the University of Western Ontario and regarded as a visitant, was found in the mantle cavity of one snail. In another, one specimen of the predatory leech *Helobdella nepheloidea* (Graf), identified by Dr. J. P. Moore of the University of Pennsylvania, was found.

A Fluke in the Uterus

In the uteri examined during the embryo counts many metacercaria were discovered. There were more in snails collected during the summer than at other times of the year and more in the snails living in the pond than in those taken from the river. The highest infestation was recorded for July 1934, when it reached 88% with an average incidence of 29 flukes per infested animal. The smallest snail dissected in this study was 15 mm. high and it contained five flukes.

Acknowledgments

The writer is indebted to the University of Western Ontario for the provision of the necessary facilities for the conduction of this study; to Professor A. D. Robertson, Head of the Department of Zoology of the University, for the direction of the work during the two years it was carried on; and to Mr. J. G. Oughton of the Royal Ontario Museum of Zoology, for assistance and advice from time to time.

References

1. Baker, F. C. Tech. Pub. N.Y. St. Coll. Forestry 16(21) : 1-366. 1916.
2. Call, R. E. Bull. Washburn Coll. Lab. Nat. History, 1(5) : 149-165. 1886.
3. Cheatum, E. P. Trans. Am. Microscopical Soc. 53 : 348-407. 1934.
4. Mattox, N. T. Am. Midland Naturalist, 16(2) : 144-153. 1935.
5. Mattox, N. T. Z. Zellforsch. mikroskop. Anat. Bd. 27, H. 4 : 455-464. 1937.
6. Prashad, B. Mem. Indian Mus. 8(4) : 253-319. 1928.
7. Van Cleave, H. J. and Altringer, D. A. Am. Naturalist, 71 : 167-184. 1937.

STUDIES ON *STRONGYLOIDES AGOUTII* SP. NOV. FROM THE AGOUTI (*DASYPROCTA AGOUTI*)[1]

By Henry J. Griffiths[2]

Abstract

A morphological and biological study is presented of a hitherto unrecorded member of the genus *Strongyloides* from the golden-rumped agouti (*Dasyprocta agouti*), a rodent native to Trinidad, B.W.I., and northern South America. The name *Strongyloides agoutii* sp. nov. is proposed for this species.

Observations on the free-living development over a period of three years showed the indirect type to prevail; no seasonal variation was observed. Continuous propagation of the free-living generation of this species was not observed in faecal cultures or on artificial media.

A brief résumé of the classical studies on species of the genus *Strongyloides* is included, together with a summary of existing hypotheses and theories on the biology of this group. A list of species and hosts for this genus is given.

Introduction

As a general rule, a bisexual parasitic generation alternating with a larval stage outside the host may be considered as characteristic of the mammalian nematode parasites. This larval stage is essential and is subject to many modifications depending upon the adaptations of the parasite concerned. The non-parasitic phase of the life cycle of *Strongyloides* is similar to that of many other intestinal nematodes in that the offspring of the parasitic generation may develop directly into forms infective to a new host. In addition to this, however, *Strongyloides* may produce a true free-living generation consisting of adult rhabditiform males and females, which in turn reproduce sexually and give rise to filariform larvae infective to a new host.

The occurrence of this true free-living generation is uncommon among nematode parasites, and the genera *Strongyloides* and *Rhabdias*, in which it is found, are differentiated from other nematodes of vertebrates by this biologically significant mode of development. Another distinguishing feature of these two genera is the fact that the parasitic stage in the intestine of the host is represented only by female worms, considered by some investigators to be parthenogenetic and by others hermaphroditic. For many years the validity of this fact was unquestioned until Kreis (23) and Faust (9) reported the occurrence of parasitic males in infections of *S. stercoralis* in man and dogs.

The genus *Strongyloides* presents excellent opportunity for detailed study of both the parasitic and free-living forms of nematodes, and offers problems in nematode biology of an intricate and complex nature.

[1] *Manuscript received September 26, 1939.*

Contribution from the Institute of Parasitology, McGill University, Macdonald College, Que., with financial assistance from the National Research Council of Canada.

[2] *Assistant.*

The studies on the genus *Strongyloides* presented in this paper were undertaken to throw further light on certain inconsistencies recorded in its life history. The free-living and parasitic phases of a hitherto unrecorded strain of *Strongyloides* were observed. The results were compared with those obtained by workers investigating other species.

Materials and Methods

The supply of *Strongyloides* material was provided by the golden-rumped agouti *(Dasyprocta agouti)*, a rodent native to Trinidad, B.W.I., and northern South America. The animals were kept indoors in wooden frame cages covered with light wire fencing. Each animal was supplied with a sleeping box and bedding of wood shavings, about 2 in. deep. The cage floor was of asphalt, which permitted easy washing and disinfection. The maintenance ration consisted of bananas and coarse rolled oats supplemented by sprouted grain, peanuts, carrots, sweet potato, or apple when available. A small amount of cod liver oil was mixed occasionally with the rolled oats as a source of vitamins A and D.

The room in which the agouti were housed was held throughout the year at a temperature of about 70° F. As the floor of the pens was covered with shavings and as the bedding was usually dry, any possibility of the propagation of various species of free-living nematodes, the introduction of which might have caused considerable confusion, was eliminated.

Owing to the rapid development of the free-living phases of *Strongyloides*, it is very desirable that faeces for culturing be collected and used as soon after passage as possible. The agouti was observed to defaecate at a fairly regular period after feeding and usually in the same location in the cage, so that collection of fresh faeces was a minor problem. When in captivity and fed on the diet detailed above, the agouti passes stools that are moist and solid.

The customary Petri dish, 100 by 15 mm., was found satisfactory for the routine faecal culture observations of the free-living phase of development. On account of the heaviness of infection of the agouti, it was not necessary to culture large quantities of faeces. If too much was used, difficulty was experienced in making accurate observations under the binocular dissecting microscope because of the density of the culture. Cultures were mixed to a semi-solid consistency and kept adequately moistened. During the early stages of observation the cultures were occasionally stirred if a fungus growth appeared. For certain observations the faecal material was mixed with approximately one-half its volume of animal charcoal.

All cultures, unless otherwise stated, were incubated at room temperature, usually 22° to 25° C., but with a possible range of from 15° to 30° C. To prevent undue drying of the cultures during incubation above 25° C., the Petri dish lids were lined with filter paper, which was readily moistened. At temperatures over 30° C., it was more satisfactory to place the cultures over water in large covered culture dishes.

The free-living and parasitic forms of *Strongyloides* were killed and fixed by various methods, and although much care and attention were given to methods employed, considerable shrinkage and distortion of the parasitic forms occurred. Specimens were killed and fixed in hot 70% alcohol containing 3 to 5% glycerine. The container was loosely covered to exclude dust and set aside until the alcohol had evaporated, thus leaving the worms in dilute glycerine, suitable for microscopic examination. This method was found to be quite satisfactory. Hot 70% alcohol was not found to be as satisfactory a fixative as the alcohol and glycerine. Most satisfactory results were obtained by killing the specimens in hot water, fixing in 5% formol-saline, washing, and gradually transferring to glycerine alcohol. Material may be stored in 70% alcohol containing 3 to 5% glycerine for a considerable time; the presence of glycerine is a safety measure against desiccation owing to the evaporation of the alcohol. The ova of *Strongyloides* can be preserved in 5% formol-saline for several months without shrinkage or appreciable distortion.

Remarks on the Genus *Strongyloides*

The homogenicity and simplicity in structure of the members of the genus *Strongyloides* may be considered responsible for the difficulties that have arisen concerning the systematics of this group. Species have been recorded from a wide variety of unrelated hosts, as shown by the following list:

List of Known Species of the Genus *Strongyloides* Grassi, 1879

Type species: *Strongyloides stercoralis* (Bavay, 1876), from man.
Other species:

S. akbari Mirza and Narayan, 1935, from *Crocidura coerula*—India.
S. avium Cram, 1929, from domestic fowl—United States.
S. canis Brumpt, 1922, from dog—Japan and China.
S. cebus Darling, 1911, from *Cebus hypoleucus*—United States.
S. chapini Sandground, 1925, from *Hydrochoerus hydrochoera*—United States.
S. felis Chandler, 1925, from cat—India.
S. fülleborni von Linstow, 1905, from *Anthropopithecus troglodytes* and *Cynocephalus babuin*—Africa.
S. longus bovis de Gaspari, 1912, from ox—Turin.
S. minimum Travassos, 1930, from *Dafila bahamensis*—Brazil.
S. mustelorum Cameron & Parnell, 1933, from *Mustela ermina*—Scotland.
S. myopotami Artigas & Pacheco, 1933, from *Myopotamus coipus*.
S. nasua Darling, 1911, from *Nasua narica panamensis*—Panama.
S. ophidae Pereira, 1929, from *Drimobius bifossatus*.
S. oswaldoi Travassos, 1930, from domestic fowl—Brazil.
S. ovocinctus Ransom, 1911, from *Antilocapra americanus*—United States.
S. papillosus (Wedl., 1856) Ransom, 1911, from sheep, goats, rabbits, etc.
S. pereirai Travassos, 1932, from *Elosia rustica*—Brazil.
S. ransomi Schwartz & Alicata, 1930, from pig—United States.

S. ratti Sandground, 1925, from *Rattus norvegicus*—United States.
S. simiae Hung and Hoeppli, 1923, from *Macaca* sp.
S. stercoralis var. *eryxi* Mirza & Narayan, 1935, from *Eryx johnii*—India.
S. stercoralis var. *vulpi* Mirza & Narayan, 1935, from *Vulpex alopex*—India.
S. suis (Lutz, 1894) von Linstow, 1905, from pig.
S. venezuelensis Brumpt, 1934, from *Rattus norvegicus*—Venezuela.
S. vituli Brumpt, 1921, from ox—France.
S. viviparous (Probstmayer, 1865) von Linstow, 1905, from horse—Europe.
S. westeri Ihle, 1917, from horse—Holland.

In addition the following undetermined species of *Strongyloides* are listed by Sandground (28):

Strongyloides sp. from lemur, Weinberg & Romanovitch, 1908.
Strongyloides sp. from fox, Romanovic, 1914.
Strongyloides sp. from guinea pig, Krediet, 1921.

Hall (20) makes reference to *Strongyloides* sp. from a rodent, but he has been unable to locate any reference to a paper by Parona whereby the observation might be confirmed.

There has been much difference of opinion as to the validity of many of these species. The difficulty of observing and recording any distinct characters on which specific distinction may be made, has led to a tendency on the part of many workers to erect new species on the grounds of the occurrence of the parasite in a previously unrecorded host. In other cases, the description of new species has been based almost entirely on dimensions of the body. These distinctions, probably legitimate in many cases, have seldom been accompanied by drawings that would be of benefit to subsequent investigators. Until recent years, there has been a tendency to neglect the bisexual forms when describing new species. In many species, the free-living forms are remarkably similar in appearance and do not possess distinctive features by which they may be characterized. However, differences do exist in the morphology of the bisexual generation, and in at least one species, *S. fülleborni*, these characters are sufficient for species identification. Goodey (15) has shown that constant differences of structure do exist between the free-living stages of *S. fülleborni* and *S. stercoralis* and that the free-living male of *S. ratti* materially differs from the free males of the other two species mentioned. These observations have been made only by examination of the species in question in as great detail as possible, together with careful camera lucida drawings. Looss (26, p. 215), who has furnished the best and most detailed drawings of *S. stercoralis*, writing on the occurrence of the genus in animals states: "Besides being found in man, species of *Strongyloides* are very frequent in animals (mammals, birds and reptiles); so far as my personal observation goes, they so greatly resemble *Strongyloides stercoralis* that it is difficult to say whether they are the same species or not. The free-living generations on the contrary, so far as I am acquainted with them, show

slight but distinct differences from one another, which make it probable that different species exist."

Chandler (4) has undertaken the comparison of representatives of the genus from monkeys, cats, and man with the published characters of other forms available at that time. He suggests that the forms of *Strongyloides* considered in his paper be grouped into two species, *S. papillosus* and *S. stercoralis*, the species *ovocinctus*, *fülleborni*, *suis*, *simiae*, and *cebus* being reduced to hostal varieties or sub-species of *papillosus*, and the species *nasua*, *canis*, and *felis* to varieties or sub-species of *S. stercoralis*.

It would appear then, that the validity of many species of *Strongyloides* is questionable. Species differentiation has in many cases been based on proportionate lengths of different parts of the body without morphological distinctions. The observations of many investigators show that there may be a very wide range of variation in these ratios even between individuals in the same host, and there is little doubt that unless accompanied by other significant morphological features, they are of no value for specific diagnosis.

At the present time, however, it would seem that lack of data prevents a systematic reorganization of the genus on a morphological, physiological, or biological basis.

Strongyloides agoutii sp. nov.

The creation of numerous species of *Strongyloides* may be unwarranted, yet, when the basis for the taxonomy of this group is eventually laid, such species can be rendered invalid with greater ease and fewer complications than if they had originally been grouped under one specific name. On these grounds, it is considered advisable to create a new species for the strain of *Strongyloides* found in the agouti *(Dasyprocta agouti)*.

A summary of the more important dimensions of 25 specimens of the parasitic and free-living generation is given in Table I. All measurements were made on unpreserved material. Specimens were examined in water and were rendered motionless by the careful application of heat.

Parasitic Generation

Female (Fig. 1). The length varies from 3.94 to 6.45 mm., and the width from 0.03 to 0.059 mm., measured at the middle of the body. The body is long and filiform, showing equal thickness from the base of the oesophagus to the region of the posterior ovarian loop. The cuticle is exceedingly finely striated, the striations being most easily seen in the tail region. From the base of the oesophagus, the body gradually becomes attenuated anteriorly to a diameter of about 0.015 to 0.020 mm. The mouth is small and leads directly into the oesophageal lumen. It consists of three indistinct lips with two projections on each, which are presumably papillae; the minute size of these structures renders them exceedingly inconspicuous. The first quarter of the oesophagus is slightly more muscular than the rest; a slight enlargement was observed at the posterior end of the first region. The oesophagus gradually increases in size posteriorly and is from 0.975 to 1.450 mm. long.

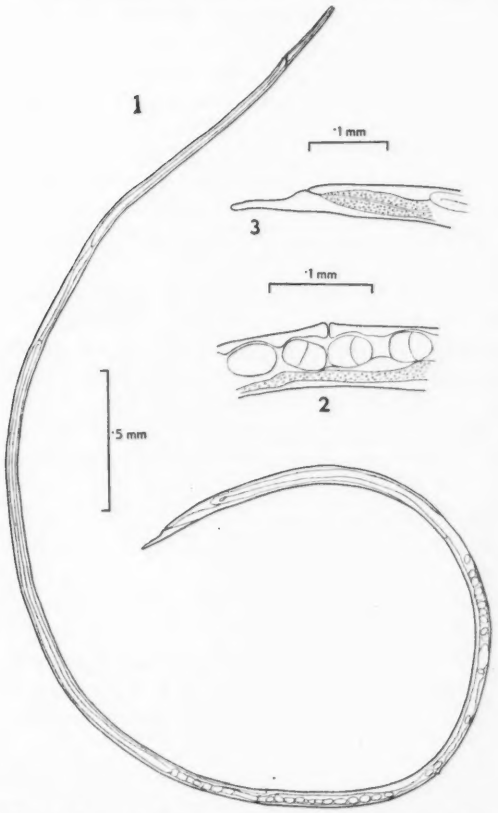
TABLE I

MEASUREMENTS OF 25 SPECIMENS OF *Strongyloides agoutii* SP. NOV.

	Range (n = 25), mm.	Mean, mm.	Standard deviation*	Coefficient of variation, %
Parasitic female				
Total length	3.94 – 6.45	5.170	0.7242	14.0
Width at middle of body	0.03 – 0.059	0.044	0.0063	14.3
Length of oesophagus	0.975 – 1.450	1.210	0.1359	11.2
Length from vulva to tip of tail	1.275 – 2.300	1.750	0.2606	14.8
Length of tail	0.075 – 0.109	0.091	0.0099	10.8
Ova—length, μ	46 – 58	51	3.926	7.6
width, μ	23 – 29	26	1.706	6.5
Free-living male				
Total length	0.870 – 1.050	0.965	0.0617	6.3
Width at middle of body	0.050 – 0.070	0.058	0.0067	11.5
Length of oesophagus	0.120 – 0.160	0.135	0.0096	7.1
Length of tail	0.075 – 0.100	0.086	0.0085	9.8
Length of spicules (10 specimens only)	0.028 – 0.035	0.031	0.0024	7.7
Length of accessory piece (5 specimens only)	0.018 – 0.023	0.021	0.0019	9.0
Free-living female				
Total length	0.975 – 1.350	1.126	0.0930	8.2
Width immediately anterior to vulva	0.045 – 0.075	0.062	0.0075	12.0
Length of oesophagus	0.140 – 0.188	0.156	0.0126	8.0
Length of tail	0.090 – 0.165	0.123	0.0170	13.7
Ova—length, μ	38 – 62	47	4.980	10.5
width, μ	28 – 40	36	3.187	8.8
Filariform larva				
Total length	0.645 – 0.760	0.710	0.0365	5.1
Width at junction of oesophagus and intestine	0.018 – 0.022	0.020	0.0012	6.0
Length of oesophagus	0.276 – 0.354	0.322	0.0252	7.7

* $\sigma = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$.

The longest oesophagus does not necessarily occur in the longest worm, and it may be said that the oesophagus varies from one-quarter to one-fifth of the total length of the body. The vulva (Fig. 2) possesses prominent lips and is situated well posterior to the middle of the body at from 1.275 to 2.300 mm. from the tip of the tail. The gonad is double and consists of an anterior and posterior loop, which may be of the simple hair-pin bend type or may be twisted. No constancy in this character was observed in the material examined. Some specimens showed the simple type in both uteri, while a few showed both loops twisted and others only one loop twisted. The ovaries and uterus occupy most of the body cavity, the bends of the former occurring close to the oesophageal and anal ends of the intestine. Between the posterior ovarian loop and the anus the body commences to taper, becoming consider-



FIGS. 1 – 3. FIG. 1. *Parasitic female.* FIG. 2. *Vulva of parasitic female.* FIG. 3. *Tail of parasitic female.*

ably reduced behind the anus and terminating in a fairly short finger-like tail, broadly rounded at the tip. The tail (Fig. 3) varies in length from 0.075 to 0.109 mm. The eggs are ellipsoidal, thin shelled, and from 46 to 58 μ long by 23 to 29 μ wide. They are not fully embryonated in the uterus, but may contain an embryo at the time of elimination with the faeces. The uteri usually contain several fully developed ova and as many as 25 undeveloped eggs may be seen.

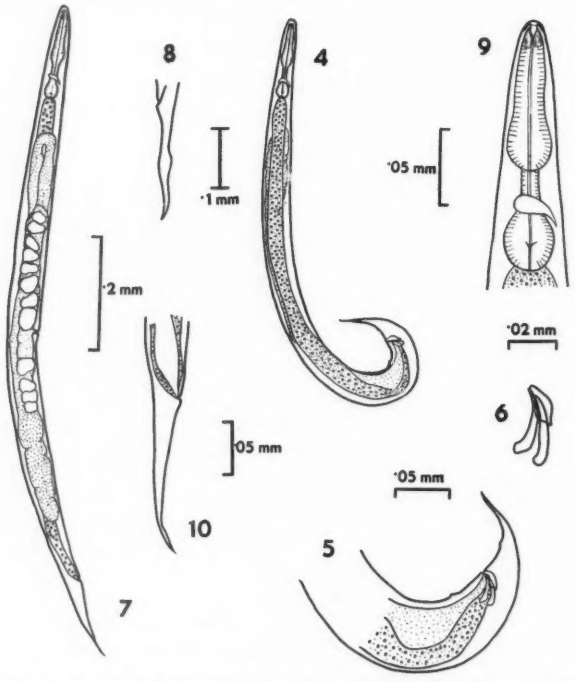
The Free-living Generation

This comprises male and female forms. The latter lay eggs that develop into rhabditiform larvae and subsequently into filariform larvae.

Male (Fig. 4). From 0.870 to 1.050 mm. in length with an average width of 0.058 mm. at the middle of the body, which is of more or less equal diameter except in the tail region. Anterior to the junction of the oesophagus and

intestine the head region tapers uniformly. Anterior to the anus the body shows marked thickening, but posterior to the opening it becomes considerably narrowed, tapering quickly and terminating in a fairly long slender point. The mouth leads into a short pharynx. The oesophagus is from 0.120 to 0.160 mm. long and is made up of two parts connected by a narrow neck. The anterior portion is more or less cylindrical with a slight swelling before narrowing to the neck region. A small area at the anterior end of the oesophagus shows a decided muscular thickening. The neck is fairly long and is followed by a round to flask-shaped, muscular bulb. The nerve ring crosses the neck just anterior to this bulb.

The male gonad is single and extends almost up to the oesophagus, occupying most of the width of the body. The terminal portion of the testis narrows considerably to form a duct leading into the cloacal opening. The tail (Fig. 5) is curved ventrally, and is from 0.090 to 0.165 mm. in length. Caudal papillae are present on the ventral surface though not very conspicuous—one pre-anal and one post-anal approximately equidistant from the anus. The spicules are 0.028 to 0.035 mm. in length and resemble a curved blade



FIGS. 4 – 10. FIG. 4. *Free-living male.* FIG. 5. *Tail of free-living male.* FIG. 6. *Accessory piece and spicules.* FIG. 7. *Free-living female.* FIG. 8. *Abnormal tail of free-living female.* FIG. 9. *Anterior end of free-living female.* FIG. 10. *Tail of free-living female.*

with a knob-like handle (Fig. 6). The accessory piece (Fig. 6) is from 0.018 to 0.023 mm. in length and is of an irregular oval shape.

Female (Fig. 7). From 0.975 to 1.350 mm. in length with an average width of 0.061 mm. immediately anterior to the vulva. Anteriorly the body tapers gradually; posteriorly the narrowing is slightly more abrupt, becoming considerably narrowed in the region of the rectum and tapering finally to a slender pointed tail. The tail was not observed to taper smoothly to a point in all cases; bulbar enlargements (Fig. 8) were observed frequently between the anus and tail tip. The head region and oesophagus of the female (Fig. 9) closely resemble those of the male. The oesophagus is from 0.140 to 0.188 mm. in length. The vulva shows well defined lips and is located slightly posterior to the middle of the body; the gonad is double and consists of anterior and posterior loops of the simple hair-pin bend type. The number of eggs present in the uteri is variable and as many as 23 were observed in well grown females. These ova are more or less sub-spherical in shape with a length of 38 to 62 μ, and a width of 28 to 40 μ. It has been observed that the female has not necessarily finished growing when eggs appear in the uterus. After oviposition is completed the uteri shrink considerably in size. The tail is slender (Fig. 10), pointed, and from 0.090 to 0.165 mm. long.

Rhabditiform larva

The rhabditiform larvae (Fig. 11) show considerable size range, depending on the stage of growth. At the time when the genital primordium is conspicuous, the larvae are approximately 0.4 mm. in length, with a width of 0.02 mm. The genital primordium is located in the region of the middle of the intestine. The tail is slender and tapering.

Filariform larva

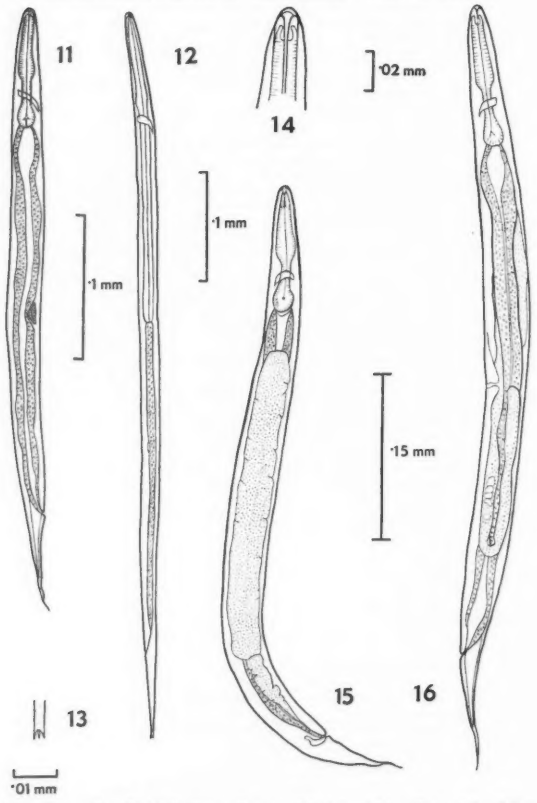
These larvae (Fig. 12) are from 0.645 to 0.760 mm. long and from 0.018 to 0.022 mm. wide in the region of the base of the oesophagus. The oesophagus is from 0.276 to 0.354 mm. long and shows a slight enlargement at the end of its first third; it joins the intestine just anterior to the middle of the body. The genital primordium is located at approximately the posterior end of the first third of the intestine. The tail is straight and tapered, showing a tricuspid termination (Fig. 13).

HOST: *Dasyprocta agouti.*

LOCATION: Small intestine.

TYPE LOCALITY: Trinidad, B.W.I.

The *Strongyloides* from the agouti differs from most of the other species on the basis of size. In this respect it falls between *S. papillosus* and *S. westeri*, though it is never as large as the latter. The majority of specimens are considerably larger than those of *S. papillosus* and the minimum lengths of oesophagus and tail are always greater than the maximum for that species. Moreover, in cross-infection experiments the agouti species will establish



FIGS. 11 – 16. FIG. 11. *Rhabditiform larva (20 hr. old).* FIG. 12. *Filariform larva.* FIG. 13. *Tricuspid tail of filariform larva.* FIG. 14. *Head of immature free-living female.* FIG. 15. *Immature free-living male.* FIG. 16. *Immature free-living female.*

itself readily in the guinea pig but not in the rabbit, whereas this condition is reversed with *S. papillosus*.

Comparative size, shape of tail, as well as the expulsion of embryonated ova in the faeces differentiated this species from *S. stercoralis*. No characteristic spear apparatus was seen in the oesophagus as found in the free-living female of *S. simiae*, and the marked post-vulvar constriction in the free-living females of *S. fülleborni* was not observed. The dimensions of the agouti species distinguish it from the much smaller *S. ratti*.

The twisting of the anterior and posterior ovarian loops does not show any constancy; and the shape of the tail of the parasitic female, although little variation is observed, cannot be considered as a distinctive characteristic.

This species, accordingly, differs from those that have been adequately described and is, therefore, referred to a new species under the name of *Strongyloides agoutii* sp. nov.

Recent Aspects on the Biology of *Strongyloides*

The parasitic generation in the genus *Strongyloides* is represented by females, considered by most authors to be parthenogenetic or hermaphroditic. These females lay eggs that may hatch within the intestine, so that free larvae are found in freshly passed faeces. In some species, however, the eggs themselves appear in the faeces. The larvae that are discharged in the faeces or hatched from the ova are known as rhabditiform larvae. These may metamorphose directly to the filariform or infective larvae or they may develop into a free-living bisexual generation of rhabditiform males and females. The free-living sexual generation produce fertile ova which give rise to rhabditiform larvae and later to infective filariform larvae. The former metamorphosis is known as the direct or homogonic type of development, the latter as the indirect or heterogonic method.

There are thus two possible cycles in the life history of members of this genus which permit alternative modes of origin of the infective filariform larvae. Since the occurrence of these two modes of development was confirmed, many hypotheses and theories concerning the factors influencing them have been offered.

Earlier workers considered that physical, chemical, and mechanical factors in association with environmental conditions, might influence the mode of development, but investigation did not confirm these views.

Recent workers, by the use of cultures in which both types had appeared, have been unable to produce changes in either type by subjection to controlled environmental conditions. The hypothesis that the direct developing type was confined to the temperate zone and the indirect to the tropics was accepted for some time (24, 25). Further suggestions were made that the differences in mode of development might be attributed to nutritional conditions to which the larvae are subjected prior to leaving the intestine of the host (Darling, 1911).

More critical methods of procedure and experimentation have proved the former theories to be untenable. Pure strains of one type were seldom observed and the general instability of types was accepted. The infection of certain abnormal hosts was found to lead to changes in the type of life cycle. Cytological studies of the gonads of the parasitic female led to the belief that the female is really hermaphroditic rather than parthenogenetic (28–31). This observation gave rise to a theory that the direction of development is determined by the chromosome constitution of the eggs after fertilization, and that the sex of the rhabditiform generation is determined according to whether the eggs are fertilized by sperm bearing, or not bearing, a heterochromosome (32).

The classical life cycle of the genus *Strongyloides* was generally accepted until comparatively recent years. In addition to the direct and indirect phases of development of *Strongyloides*, a distinct hyper-infective type was stated to occur, resulting from the rhabditiform larvae metamorphosing to

infective larvae prior to feeding (6, 7). This type is, therefore, the form responsible for the so-called "auto-infection" or "hyper-infection" of individuals that have become parasitized previously. At the same time, the administration of gentian violet as a strongyloidicide was believed to change the indirect and hyperinfective strains of *Strongyloides* to the direct type, a factor of no little importance to the fundamental biological ideas of the life cycle of the genus (8).

The discovery of male parasitic stages gave rise to further hypotheses concerning the mode of development of the free-living generation (9, 23). Evidence has been presented in support of the view that spermatozoa observed in the reproductive tubule of the female are the result of a process of insemination by a male. It is suggested that this process occurs during the latter part of the adolescent stage of the female, and that the insemination may take place in the lungs (where females and males have been observed together) prior to the migration of the female into the mucosa of the intestinal wall. This insemination is considered to suffice for several months after the females invade these tissues. The belief that fertilized ova give rise to an indirect type, while unfertilized ova may produce a direct type of development, was put forward. This idea is consistent with the tendency of strains that have dwelt within a host for any length of time to change from indirect to direct type as the supply of spermatozoa becomes depleted.

Experimental evidence has been presented to show the continued propagation of the free-living generation on artificial media (1, 2). It has been stated that when a known species of the genus, which most nearly demonstrates a typical indirect mode of development, is cultured under optimum conditions, it will continue to develop indirectly. However, when subjected to unfavourable environmental conditions (e.g., reduction in quantity or quality of nutrient or reduction in viscosity) a modification of the strain toward a direct type may be observed. Free-living adults of the first, second, and third generations were grown on artificial media; and it is believed that continued propagation of the free-living sexual generation occurs normally in nature under optimum conditions. This evidence would tend to suggest that "directness" and "indirectness" are conditioned by environment and not by the "genetical make up" of the ovum.

The biological studies on the genus during the past few years have tended to complicate rather than simplify the picture. As a result of critical investigations involving the transfer of a pure line strain of *S. ratti* through 14 parasitic generations by means of single larva transfers, it has been demonstrated that a male parasite is unnecessary in the bionomics of *S. ratti* either to maintain parasitic fertility or to explain the two modes of larval development (16–18). The theory of hyper-infection as a possible source of either hypothetical parasitic males or additional parasitic females is not supported, and the parasitic phase of *S. ratti* is considered to be represented by a female organism either syngonic or parthenogenetic.

It has recently been shown that the frequency with which the daily yield of offspring (resulting from singly established parasites of *S. ratti* in the rat) included adults of heterogonic development was subject to fluctuations over long seasonal cycles. Data suggest that the rat responds physiologically to changing meteorological conditions and that the altered environment thus produced for *S. ratti* leads to changes in the relative frequency with which progeny of heterogonic development are produced. All evidence from these recent studies indicates that the mode of larval development shown by the progeny of *S. ratti* is determined prior to oviposition (19).

The Free-living or Sexual Generation of *Strongyloides agoutii*

It was considered advisable to continue observations of development of the sexual generation over as long a period as possible, in order to record any appearance of latent factors that might not reveal themselves in a short time. The observations extended from April 1936 until March 1939, some 139 cultures being made. The collection of at least three faecal samples per month seemed desirable, and as a general rule these were collected on different days; on occasions when cultures failed to furnish a sufficient nematode population, further samples were collected during that month. In this way, it was considered that any seasonal variation in development would be observed.

All cultures during 1936 were kept at room temperature, which ranged from 15 to 30° C., with a usual temperature of 23° C. During the first six months of 1937 the temperature was held regularly at 22° C., though on 15 occasions in May and June it ranged between 25 and 30° C. During the latter part of 1937 and throughout 1938 and 1939 the temperature variation was similar to 1936.

The routine procedure adopted in examination was modified to suit each set of cultures. However, since with this species ova are deposited in the faeces, the first examination of all cultures was made on the morning following the day of collection, usually 20 hr. after passage; subsequent examinations were daily or oftener, as necessary. If only filariform larvae were present on two or more consecutive examinations, the daily observations were discontinued and cultures were examined on alternate or on every third day. As the Petri plate lids were not lined with filter paper, it was usually necessary to moisten cultures at least once a week. Sufficient aeration was provided by removal of the lids when examination was carried out under the binocular microscope. In most instances, developmental forms could be differentiated under 16 to 32 magnifications, but if doubtful they were examined under the compound microscope. Cultures were discarded when two or more consecutive examinations showed no activity.

It will be observed that the total number of cultures recorded on the graphs does not correspond to the total examined in the investigation. A few records have been deleted when the culture has been rendered inactive soon after

origin, because of abnormal conditions; in some, larval development was not observed; in a few, all the larvae were used for experimental infection purposes. During the three-year period, time lost by sickness or holidays was responsible for the discard of some cultures. The analysis of these cultures shows the indirect or heterogonic type of development was consistent over the three years, showing no variation from month to month.

As a general rule, rhabditiform larvae were not observed on first examination of the faeces. In the majority larvae were not observed in any number for 48 hr., after which immature males and females were observed. In all instances females predominated in numbers, males being present in most but always in a great minority. The length of life of the male was considerably shorter than that of the female, the former dying after the second or third day of activity.

From Fig. 17 it will be seen that most free-living sexual forms appeared between the second and fifth days of culture and disappeared over a period of from 3 to 11 days. Free-living sexual stages disappeared from most cultures by the seventh or eighth day, although in four cases they lived as long as 11 days.

In Fig. 18 the first appearance of filariform larvae is indicated. In many cultures filariform larvae appeared on the fourth day, though in the greatest number they appeared on the sixth day; by the eighth day, larvae were present in all cultures.

The criterion of life of cultures is the activity of filariform larvae. From Fig. 19 it is seen that in the greatest number of cultures larvae became inactive between the 21st and 25th days. They remained active in some for 35 to 40 days, and in one only for 41 days. From Fig. 18 it is seen that filariform larvae appeared in the majority by the sixth day. Accordingly, the maximum duration of life of the filariform larvae is 35 days.

The Continued Propagation of the Free-living Generation of *Strongyloides agoutii*

The first intensive study of this nature with a member of the genus *Strongyloides* was made by Beach (1, 2). With both human and primate species of *Strongyloides*, he recorded continued propagation of the free-living phase of *S. simiae* on artificial culture media for three generations. His extensive investigations show that *S. simiae*, which follows the totally indirect type of development, changed to a mixed type when cultured on various artificial media. Parthenogenesis in the free-living female of this species was not found to occur. A single parasitic female, placed in half-strength Locke's solution, laid eggs that developed into males and direct filariform larvae in three cultures, while males, females, and filariform larvae developed in another.

Observations on the filariform larvae of *S. fülleborni* in different media as well as in tissue culture were recorded by Chung (5). In most instances there was no development, though he found that larvae survived for many

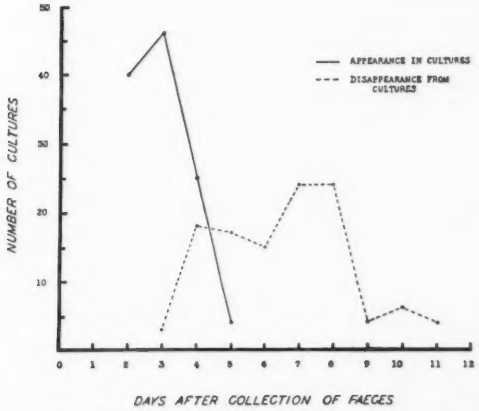


FIG. 17. *Appearance and disappearance of free-living sexual stages of S. agoutii in faecal cultures from agouti.*

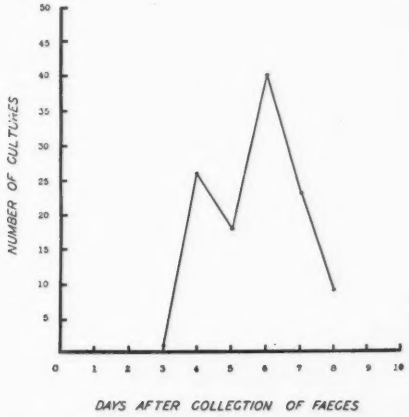


FIG. 18. *Appearance of filariform larvae of S. agoutii after passage of faeces by agouti.*

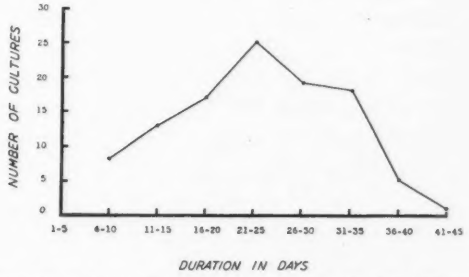


FIG. 19. *Longevity of filariform larvae of S. agoutii in faecal cultures from agouti.*

days in a medium having a pH between 5.4 and 7.0; a pH above 7.8 was unfavourable. Kouri, Basneuvo, and Arenas (22) report that with *S. stercoralis*, after numerous free-living generations, a change to an entirely free-living individual takes place, the female becoming parthenogenetic and there being no males. The fecundity of these females gradually decreases until the cultures become sterile. The findings of Beach (2) indicate that directness and indirectness in development of the free-living phase of the life cycle is contingent on environmental factors only. He considers that optimum conditions tend to produce the indirect type of development, whereas unfavourable conditions produce the direct.

In order to ascertain if continuous propagation of *S. agoutii* could be induced in artificial culture, a series of experiments was made.

To permit detailed observation of isolated forms in culture, a glass culture slide with a well 16 mm. in diameter by 3 mm. deep was employed. Small Petri dishes were also found satisfactory when it was desired to culture several individuals together. The medium used was similar to that used by Beach, with substitution of extract of agouti faeces in place of monkey faeces, and 1 gm. instead of 2 gm. of agar. It consisted of the following: 1 gm. of nutrient agar, 25 cc. of aqueous extract of agouti faeces, 75 cc. of distilled water. It was sterilized at 15 lb. for 20 min. to ensure the killing of any ova or larvae that might have been present. A thin film was placed on the bottom of the Petri dishes or of the well in the slides. This thin layer permitted the use of the compound microscope for examination purposes.

Larvae were obtained by Baermanning cultures of agouti faeces. Mature males and females were then selected. No attempt was made to control bacterial development in the cultures, but colonies were observed in only a few instances. It was necessary to moisten cultures quite frequently even though the slides were kept over water in large glass culture dishes, the lids of which were lined with filter paper to avoid excessive evaporation. Cultures were examined daily. Some 30 isolation cultures were made and examined in sets of six. The first group was cultured at room temperature of approximately 23° C. Slides No. 1, 2, and 5 were allotted two mature females each; No. 3, one immature female; No. 4, one female and one male; and two males to No. 6. With the exception of No. 1 and 4, the free-living stages were all dead by the fourth day; No. 1 and 4 showed active filariform larvae until the 18th and 10th days respectively.

Group 2 was cultured at 30° C. Slides were prepared with the following combinations: No. 7 with one male; No. 8 and 9 with a female each; No. 10 with a female and a male; and No. 11 and 12 with two females each. All individuals in this group were dead by the fourth day and no progeny was recorded.

Group 3 was cultured at room temperature. Slides No. 13–15 were made up with one female and one male each; No. 16 and 17 with two females each; No. 18 with two males. The males on No. 18 were dead by the third day.

Filariform larvae were observed in the other cultures and the duration of activity noted was 5, 6, 7, and 18 days.

Group 4 was cultured at 25° C. Slides No. 19–22 were allotted one male and one female each. No. 19 showed active filariform larvae up until the 11th day; No. 21 showed larvae until the fifth day; No. 20 and 22 produced no progeny. Progeny of No. 23, which comprised two females, were active until the 18th day; and the two males of No. 24 lived but three days.

Small Petri plates were used for cultures No. 25 to 30. The first three contained five females and one male each, the others, five females and five males each. The cultures were kept at room temperature (23° C.) and filariform larvae developed, but all activity ceased on the seventh and eighth days.

Discussion

A morphological and biological study of a hitherto unrecorded member of the genus *Strongyloides* has justified the creation of a new species, *S. agoutii*.

Variation in rate of growth and maturity of free-living forms was observed to occur, the regulating factor probably being food supply or environmental conditions of the media. The wide range of results, presented graphically, was unavoidable both on account of the actual error in observation and the excessive rate of metamorphosis of forms from one stage to another.

The frequency of the first appearance of filariform larvae on the fourth day might possibly be interpreted as the appearance of direct development in cultures. This occurrence could conceivably have been overlooked or not differentiated by the technical methods employed in routine observations. However, since the direct type of development was never observed, it seems improbable that it occurred.

From the data obtained, there is no evidence of continued propagation of the free-living generation of *S. agoutii*.

Acknowledgment

This investigation was undertaken at the Institute of Parasitology, Macdonald College, under the supervision of the Director, Prof. T. W. M. Cameron, who brought the agouti from Trinidad. The author wishes to express his appreciation and thanks to Professor Cameron for his continued interest, guidance, and generous assistance at all times throughout this study.

Bibliography

1. Beach, T. D. Proc. Soc. Exptl. Biol. Med. 32 : 1484-1486. 1935.
2. Beach, T. D. Am. J. Hyg. 23 : 243-277. 1936.
3. Brumpt, E. Compt. rend. soc. biol. 85 : 149-152. 1921.
4. Chandler, A. C. Parasitology, 17 : 426-433. 1925.
5. Chung, H. L. Z. Parasitenk. 9 : 28-49. 1936.
6. Faust, E. C. Proc. Soc. Exptl. Biol. Med. 28 : 253-255. 1930.
7. Faust, E. C. Proc. Soc. Exptl. Biol. Med. 28 : 919-920. 1931.
8. Faust, E. C. Am. J. Hyg. 14 : 203-211. 1931.

9. FAUST, E. C. Am. J. Hyg. 18 : 114-132. 1933.
10. FAUST, E. C. and KAGY, E. S. Am. J. Trop. Med. 13 : 47-65. 1933.
11. FÜLLEBORN, F. Arch. Schiffs-u. Tropen-Hyg. 25 : 121-123. 1921.
12. FÜLLEBORN, F. Arch. Schiffs-u. Tropen-Hyg. 28 : 144-165. 1924.
13. FÜLLEBORN, F. Arch. Schiffs-u. Tropen-Hyg. 30 : 732-749. 1926.
14. FÜLLEBORN, F. Arch. Schiffs-u. Tropen-Hyg. 30 : 721-732. 1926.
15. GOODEY, T. J. Helminthol. 4 : 75-86. 1926.
16. GRAHAM, G. L. Am. J. Hyg. 24 : 71-87. 1936.
17. GRAHAM, G. L. Am. J. Hyg. 27 : 221-234. 1938.
18. GRAHAM, G. L. J. Parasitol. 24 : 233-243. 1938.
19. GRAHAM, G. L. Am. J. Hyg. D, 30 : 15-27. 1939.
20. HALL, M. C. Proc. U.S. Natl. Museum, 50 : 1-258. 1916.
21. HUNG, SEE LU and HOEPPLI, R. Arch. Schiffs-u. Tropen-Hyg. 26 : 118-129. 1923.
22. KOURI, P., BASNEUVO, J. C., and ARENAS, R. Rev. Parasitol. Clin. y Lab. 2 : 1-6. 1936. (Helminthol. Abstr. 5 : 2 : No. 156a. 1936.)
23. KREIS, H. A. Am. J. Hyg. 16 : 450-491. 1932.
24. LEICHTENSTERN, O. Deut. med. Wochschr. 8 : 3-13. 1898.
25. LEICHTENSTERN, O. Centr. Bakt. Parasitenk. I. Abt. 25 : 226-231. 1899.
26. LOOSS, A. Rec. Egypt. Govt. Sci. Med. Cairo, Vol. 4. 1911.
27. NISHIGORI, M. J. Formosa Med. Soc. No. 277 : 1-56. 1928. (Trop. Diseases Bull. 25 : 962.)
28. SANDGROUND, J. H. J. Parasitol. 12 : 59-82. 1925.
29. SANDGROUND, J. H. 14th Ann. Rept. United Fruit Co. 240-245. 1925.
30. SANDGROUND, J. H. Am. J. Hyg. 6 : 337-388. 1926.
31. SANDGROUND, J. H. Am. J. Hyg. 8 : 507-538. 1928.
32. SCHUURMANS STEKHOVEN, J. H. JR. Z. Parasitenk. 1 : 231-261. 1928.

CANADIAN WILTSHIRE BACON

V. QUANTITATIVE BACTERIOLOGICAL STUDIES ON CURING PICKLES[1]

By N. E. Gibbons[2]

Abstract

Five combinations of diluent, salt concentration of medium, and incubation temperature have been used to study the bacterial content of curing pickles used for making Wiltshire bacon. On representative pickles from 16 plants the highest mean count was on 10% salt agar (brine dilution) incubated at 20° C. The lowest count was on nutrient agar incubated at 37° C. Counts on media containing no salt, 4% salt, and 10% salt (water dilution) with incubation at 20° C. gave intermediate values. Counts on spent pickles were higher than on cover pickles. Pump pickles showed a surprisingly high number of organisms.

The analytical error attributable to diluting, plating, and counting was relatively small compared with the other sources of variance. Under certain conditions the error of sub-sampling a small jar of pickle exceeds the error between replicate plates. Sampling and sub-sampling errors were therefore the primary factors limiting the precision of the determinations.

Statistical analysis of the results showed that the differences in numbers observed by the different methods were highly significant for all pickles from 16 plants, and that the bacterial content of the pickles from the different plants differed significantly over all media. The number of bacteria observed by the different methods was usually correlated, i.e., pickles from a plant showing a high count by one method usually showed a high count by the other methods and vice versa. Nevertheless, it was possible to demonstrate a significant differential response of the bacteria in cover and spent pickles to the different growth conditions used. This suggests qualitative differences in the flora of the pickles. Of the several growth conditions tested, nutrient agar at 20° C. and 10% salt agar with brine dilutions appear to be the most suitable for demonstrating differential responses attributable to qualitative differences between the flora of different pickles.

Introduction

An outline of the investigation on Wiltshire-cured bacon has been given in a previous paper (2). The present paper deals with the quantitative bacteriological findings on representative curing pickles obtained from the various plants. Several combinations of growth conditions were used in order to obtain, in addition to the strict quantitative variations in bacterial populations, an estimate of the validity of this procedure for detecting qualitative differences in the bacterial flora of different plants.

Samples of the three pickles used for curing were obtained from each plant. The pump pickle, injected into the sides prior to cure, is usually a freshly prepared brine which might reasonably be expected to contain few bacteria. The tank pickle, used to cover the sides during cure, was analysed at the beginning and end of cure, these samples being designated "cover" and "spent" pickles respectively. Details of the design of the experiment and methods of

[1] *Manuscript received February 9, 1940.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Issued as Paper No. 38 of the Canadian Committee on Storage and Transport of Food. N.R.C. No. 905.

[2] *Bacteriologist, Food Storage and Transport Investigations.*

sampling and of shipping the pickle have been described previously (2). Most pickles were sampled within an hour or two of receipt, although occasionally it was necessary to hold them as long as 12 hr. Pump pickles from 19 plants, and cover and spent pickles from 16 plants, furnished the data on which this paper is based.

Statistical methods were used to reduce and assist in the interpretation of the data (6). Since the bacterial numbers showed great variation, both between plants and the various cultural methods employed, the logarithms of the numbers were used both for convenience and valid interpretation on a statistical basis (1).

Cultural Methods

Lochhead (5) has shown that the count obtained on nutrient agar with incubation at 37° C., as commonly used in most packing plants for control purposes, is lower than that on nutrient agar or salt agar incubated at 20° C. Nutrient agar containing 10% salt, following dilution in brine of the same concentration, gave the highest counts, these being about 80 times greater than those obtained on nutrient agar incubated at 37° C. Water dilutions were found to render up to 90% of the organisms in pickle incapable of growth.

TABLE I

METHODS USED TO ESTIMATE THE NUMBER OF BACTERIA IN CURING PICKLES

Method	A	B	C	D	E
Temp. of incubation, °C.	37	20	20	20	20
Diluent	Dist. water	Dist. water	Dist. water	4% NaCl*	10% NaCl*
Medium	Beef-extract agar	Beef-extract agar	Beef-extract agar + 10% NaCl	Beef-extract agar + 4% NaCl	Beef-extract agar + 10% NaCl
Period of incubation, days	3	7	10	7	10

* % = *Gm. added to 100 ml. of liquid.*

These facts formed the basis for adopting the methods outlined in Table I. Method A was selected since it is commonly used for control purposes in packing plants. As a working hypothesis it was assumed that Method B would yield some estimate of the organisms introduced by the fresh sides; Method C, the salt-tolerating organisms capable of withstanding considerable change in osmotic pressure; Method D, the organisms capable of rapid growth on cured bacon; and Method E, the halophilic flora of the pickle. It is recognized that these methods may overlap considerably and that they do not yield an accurate estimate of the various types present. They were, however, considered suitable for a preliminary investigation of the subject.

Sampling

Since it was impossible to visit each of the plants, the samples were taken by a plant operator, who was provided with detailed instructions. By obtaining two samples of the pickle (taken at different times) from each plant,

it was possible to obtain an estimate of the combined error due to sampling and to any differential change that occurred in the samples during shipment. A discussion of this phase of sampling has been given (2).

Apart from any systematic change that may occur in the bacterial numbers during shipment at different temperatures over varying periods of time, it is of interest to point out that a sub-sampling error arising from the removal of duplicate samples from a small jar of pickle was greater in some instances than the analytical error of plating and counting. The results of typical experiments appear in Table II. It can be seen that the sub-sampling error is significantly greater than the analytical error in two experiments at 22° C. and approaches significance in the third. Of the six experiments at 1.1° C., three of which were shaken more frequently during storage than the others, in only two was the sub-sampling error significantly greater. In both instances the variance necessary for significance was contributed by one set of duplicates. Since this error is apparently greater when pickle is stored at the higher temperature, it is possible that it is the result of protein precipitation which interferes with the uniform distribution of the organisms through the sample.

TABLE II

SAMPLING AND ANALYTICAL ERRORS OF COUNTS ON PICKLES STORED UNDER DIFFERENT CONDITIONS

Pickle	Method	Storage temperature, °C.	Sampling and analytical error		Analytical error		*F*
			D.f.	Mean sq.	D.f.	Mean sq.	
1	B	22	7	.0817	14	.0062	13.18**
		1.1	6	.0178	12	.0083	2.14
		1.1 (shaken)	6	.0148	12	.0099	1.49
2	B	22	7	.0249	13	.0094	2.65
		1.1	6	.0021	12	.0041	0.51
		1.1 (shaken)	6	.0047	12	.0012	3.77*
	E	22	7	.0378	28	.0044	8.59**
		1.1	6	.0086	24	.0027	3.18*
		1.1 (shaken)	6	.0091	24	.0177	0.51

* *Indicates 5%,* ** *1% level of significance.*

Quantitative Results

Typical arithmetic counts of cover pickles by the various methods are shown in Table III. The logarithms of the mean, maximum, and minimum number of bacteria per ml. over all plants are given in Table IV. In general the counts were maximal on 10% salt agar (Method E) and decreased with decreasing salt concentration (Methods D and B) and with increasing temperature (Method A). Dilution with water and incubation on 10% salt medium (Method C) gave results comparable with Method B. The mean

TABLE III

REPRESENTATIVE BACTERIAL COUNTS ON COVER PICKLES UNDER DIFFERENT CULTURAL CONDITIONS. NUMBER OF ORGANISMS PER ML.

Sample	Dist. water nut. agar 37° C. (A)	Dist. water nut. agar 20° C. (B)	Dist. water 10% salt agar 20° C. (C)	4% brine 4% salt agar 20° C. (D)	10% brine 10% salt agar 20° C. (E)
1	700	5,000	7,400	60,000	900,000
2	75,000	96,000	57,000	300,000	470,000
3	12,000	36,000	19,000	61,000	120,000
4	46,000	150,000	170,000	900,000	4,700,000
5	70	600	500	770	600
6	630	12,000	11,000	30,000	34,000
7	39,000	130,000	63,000	500,000	490,000

TABLE IV

BACTERIAL COUNTS OF CURING PICKLES UNDER DIFFERENT CONDITIONS OF GROWTH

	Logarithm of number of organisms per ml.				
	Dist. water nut. agar 37° C. (A)	Dist. water nut. agar 20° C. (B)	4% brine 4% salt agar 20° C. (D)	10% brine 10% salt agar 20° C. (E)	Dist. water 10% salt agar 20° C. (C)
Pump pickle					
Mean	3.01	3.69	4.25	4.40	3.78
Maximum	4.65	4.89	5.40	5.98	4.89
Minimum	1.68	2.40	2.43	2.15	2.13
Stand. dev.	.71	.65	.88	1.10	.78
Coeff. of variation	23.6	17.6	20.7	25.0	20.6
Cover pickle					
Mean	3.63	4.36	4.96	5.33	4.29
Maximum	5.00	5.36	6.24	7.12	5.48
Minimum	1.14	2.11	2.23	2.46	2.21
Stand. dev.	1.09	.87	1.07	1.23	.87
Coeff. of variation	30.0	20.0	21.6	23.1	20.3
Spent pickle					
Mean	4.44	4.99	5.68	5.95	4.88
Maximum	5.22	5.78	6.89	7.15	5.87
Minimum	3.26	3.85	4.35	4.47	3.68
Stand. dev.	.51	.44	.56	.66	.51
Coeff. of variation	11.5	8.8	9.9	11.1	10.4

number of bacteria in cover and spent pickles from all plants shows that Methods A, B, and D measure about 2.5, 10, and 50%, respectively, of the number observed by Method E. This agrees quite well with Lochhead's figures of 1.3, 5.7, and 52.3% obtained from a number of observations on the curing pickle of one plant (5).

On all media the mean count increases in the order, pump, cover, and spent pickle. It is interesting to note that the freshly prepared pump pickle contains, on the average, almost as many organisms as the cover pickle. In fact,

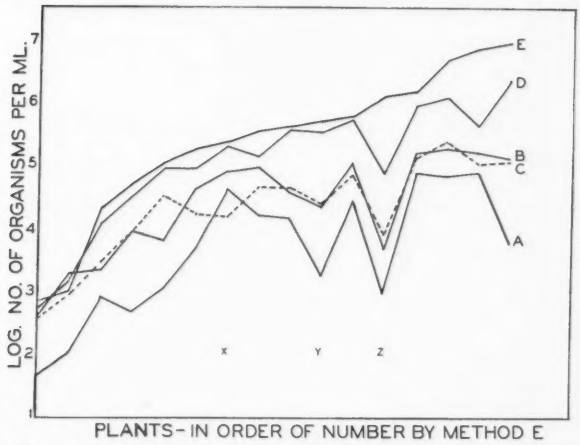


FIG. 1. *Bacterial content of cover pickle from 16 plants as indicated by different cultural methods: A—nutrient agar incubated at 37° C.; B—nutrient agar incubated at 20° C.; C—10% salt agar from water dilutions; D—4% salt agar; and E—10% salt agar from brine dilutions. All incubated at 20° C.*

the minimum values for pump pickle are usually higher than those for cover pickle.

The maximum and minimum values, the standard deviation and the coefficient of variation (Table IV) give some indication of the variation that occurs between the various counts and pickles. Since the standard deviation usually increases with the mean number present by each method, the coefficient of variation is relatively constant for each type of pickle. The standard deviation and coefficient of variation indicate that counts on nutrient agar at 20° C. are the least variable, both absolutely and relatively, while the 10% salt agar count is relatively the most variable for pump pickle and the 37° C. count relatively most variable for cover and spent pickles. It is evident that the spent pickle is the least variable of the three types. It would seem that the number of bacteria present in cover pickle reflects the many variations in pickle formula and plant practice, but during cure becomes more uniform.

Sources of Variability

The variability indicated by the standard deviation in Table IV can be divided into that originating from three main sources: (i) combined error of dilution, plating, and counting (analytical error); (ii) combined error of sampling the tank, differential changes during shipment, and sub-sampling the jar of pickle (sampling error); and (iii) the difference in count of pickles from different plants. Since the number of organisms in the pickle may vary from time to time in the same plant, the sampling error is probably overestimated.

The analysis of variance of the counts on the three types of pickle is shown in Table V. In all cases the sampling error was significantly greater than

TABLE V

ANALYSIS OF VARIANCE OF BACTERIAL COUNTS ON PICKLES

Variance	Dist. water nut. agar 37° C. (A)		Dist. water nut. agar 20° C. (B)		4% brine 4% salt agar 20° C. (D)		10% brine 10% salt agar 20° C. (E)		Dist. water 10% salt agar 20° C. (C)	
	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.
Pump pickle										
Analytical error	71	.0042	72	.0014	74	.0020	72	.0023	74	.0020
Sampling error	19	1.1564**	19	.4593**	19	.3645**	19	.5663**	19	.5337**
Between plants	18	1.8093	18	2.0360**	18	4.3358**	18	6.7464**	18	3.1623**
Cover pickle										
Analytical error	64	.0142	61	.0022	62	.0012	61	.0026	61	.0034
Sampling error	16	1.1579**	16	.6007**	16	.2541**	16	.8702**	16	.8003**
Between plants	15	6.3017**	15	4.0321**	15	6.0120**	15	8.2778**	15	3.7652**
Spent pickle										
Analytical error	59	.0019	62	.0028	61	.0049	61	.0056	61	.0016
Sampling error	16	.3377**	16	.2597**	16	.2468**	16	.2741**	16	.2958**
Between plants	15	1.2070**	15	.9020**	15	1.6128**	15	2.3336**	15	1.2660**

** *Indicates 1% level of significance.*

the analytical error. Most of the variance attributable to sampling was contributed by a few plants showing considerable differences between the two samples. In spite of the large sampling error the difference between the bacterial numbers present in the pickles from different plants was highly significant in all but one instance. It can therefore be said with assurance that the bacterial numbers present in the pickles vary much more between plants than within plants, since the latter source of variance is included in the sampling error.

Relations of Counts by Various Methods

The numerical differences observed by the various methods may result from (i) essentially similar floras in all pickles with a fixed proportion of the maximum observed population developing under the different conditions, or (ii) different floras with a different fraction of the total population favoured by each method. Obviously a high degree of correlation between the quantitative counts by the several methods would indicate the first condition, while no correlation would suggest the second. Preliminary examination of the data indicated that there was some evidence favouring both of the above behaviours. For instance, a pickle having a high count by one method usually had high counts by all methods, suggesting a correlation between them. On the other hand, although the number of bacteria observed on nutrient agar incubated at 37° C. was on the average 2.5% of that observed on 10% salt agar incubated at 20° C., for the individual pickles this proportion varied from 0.08 to 16%. Although a general association exists between the bacterial numbers observed by the different methods for each cover pickle,

TABLE VI

CORRELATION BETWEEN NUMBER OF BACTERIA GROWING ON VARIOUS MEDIA

Quantities correlated			Correlation coefficient (r)		
			Pump pickle[1]	Cover pickle[2]	Spent pickle[2]
Number on nut. agar 20° C.	*and*	Number on nut. agar 37° C.	.92	.94	.85
		4% salt agar	.89	.91	.90
		10% salt agar	.86	.86	.77
		10% salt agar (water)	.94‡	.93	.92
Number on 4% salt agar	*and*	Number on nut. agar 37° C.	.74†	.90	.81
		10% salt agar	.97‡	.91	.81
		10% salt agar (water)	.94‡	.96	.91
Number on 10% salt agar	*and*	Number on nut. agar 37° C.	.72†	.81	.70
		10% salt agar (water)	.94‡	.92	.83
Number on nut. agar 37° C.	*and*	Number on 10% salt agar (water)	.83	.87	.80

[1] *17 degrees of freedom, r of .58 required for 1% level of significance.*
[2] *14 degrees of freedom, r of .62 required for 1% level of significance.*
‡ *Significantly different from* †.

as shown in Fig. 1, there are some wide fluctuations as indicated when the numbers obtained from the plants marked y and z are compared with those from plant x.

In order to place these observations on a more quantitative basis, correlation coefficients were computed between the numbers of bacteria observed by the different methods. The values obtained appear in Table VI. All of these coefficients are statistically significant, thereby demonstrating a definite association between the numbers of bacteria indicated by the different methods. In other words, a pickle having relatively large bacterial numbers by one method will generally have relatively large numbers by all methods. On the other hand, the magnitude of the correlation coefficients is such that the residual variance unaccounted for by the coefficient varies from 5 to 50% of the total variance. This shows that there is a considerable element of independent fluctuation between the numbers observed by the different methods. These independent fluctuations responsible for the residual variance may be attributable wholly, or in part, to experimental error, or, as suggested earlier, to the possibility that the various cultural methods are capable of distinguishing to some extent a real difference between the floras present in different pickles. In order to determine the significance of these observations it was necessary to determine the differential response of the bacterial population to different cultural methods in a manner which permitted comparison with the known experimental errors. This suggested a variance and regression analysis of the data.

The differential response of the flora in the different pickles to the different cultural conditions is included in the interaction mean square, plants × methods (Table VII). This interaction should be tested for significance by comparing it with the variance between aliquots. Since duplicate determinations were not made on each medium this comparison was impossible. However, it has been shown (Table II) that for pickles kept at low temperatures the error between aliquots (sampling and analytical error) was seldom significantly greater than the analytical error alone, and in consequence the largest analytical error observed was used for comparison. On this basis the interaction mean square was found to be overwhelmingly significant.

TABLE VII

VARIANCE AND REGRESSION ANALYSES OF COUNTS BY ALL METHODS

Nut. agar 37° C.; nut. agar, 4% and 10% salt agar at 20° C.; 10% salt agar from distilled water at 20° C.

Variance	Pump		Cover		Spent	
	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.
Between plants	18	15.9807**	15	11.3190**	15	6.3109**
Between media	4	33.5442**	4	94.6674**	4	36.2393**
Differential plants × media	72	.6703**	60	.7307**	60	.3137**
Greatest analytical error	71	.0042	64	.0142	61	.0056
Greatest sampling error	19	1.1564	16	1.1579	16	.3377
Regression						
Differences in regressions	18	12.2352**	15	8.4022**	15	3.5119**
Residuals	57	1.2185	48	2.8537	48	1.2554

** *Indicates 1% level of significance.*

The fact that this plants × media interaction derived from an analysis of variance was statistically significant does not provide definite evidence that the organisms present in the different pickles respond differentially to the different growth conditions. The magnitude of the interaction may be affected by the number of organisms present in the pickle as well as by differential effects attributable to differences in flora. Since the number of organisms in the different pickles has been shown to differ significantly, it is necessary to determine the true differential effect from a regression analysis (7). It was then found (Table VII) that the differences between regressions accounted for the major portion of the interaction variance in pump pickle. There is, therefore, little evidence to indicate that different types of floras are present in this pickle. For cover and spent pickles the variance due to differences in regressions, although significant, accounts for only about half the interaction variance. The remainder is therefore attributed to differential response of the organisms to different growth conditions. This finding suggests some difference in the types of organisms present in the different pickles.

The various cultural methods included the effect of salt concentration, temperature of incubation and dilution with water and 10% brine. A further study was made to determine which of these factors contributed the most variance to the observed interaction (Table VII). The results, appearing in Table VIII, show that an increase in salt concentration from 0 to 10% in the medium contributes significantly more to the interaction than increasing the incubation temperature from 20° to 37° C. with a salt-free medium. The effect of dilution method (water or 10% brine) has a smaller differential effect than the salt concentration in the medium but a somewhat greater effect than the incubation temperature.

TABLE VIII

INTERACTIONS OF PAIRED METHODS SHOWING EFFECT OF TEMPERATURE OF INCUBATION, AND SALT CONTENT OF MEDIUM AND DILUENT

		Effect of					
		Temperature of incubation		Salt conc. of medium		Salt conc. of diluent	
		D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.
Pump	Interaction	18	.1722†	18	1.2458‡	18	.6294‡
	Greatest anal. error	71	.0042	72	.0023	72	.0023
Cover	Interaction	15	.4259†	15	1.2377‡	15	.9579
	Greatest anal. error	64	.0142	61	.0026	61	.0034
Spent	Interaction	15	.1650†	15	.5304‡	15	.9553‡
	Greatest anal. error	62	.0028	61	.0056	61	.0056

All interactions significant to 1% level.
‡ *Significantly different from* †.

These results show that the bacteriological difference between corresponding pickles from different plants is mainly quantitative. Cover and spent pickles, however, also show some differential response when cultured by different methods, suggesting qualitative differences in the flora. Media containing no salt and 10% salt incubated at 20° C. appear to be the most effective for demonstrating this differential response.

These findings are in agreement with practical conclusions. Since pump pickle is made in all plants from similar ingredients, quantitative rather than qualitative differences might be expected between different plants. However, because of the many methods of reclaiming spent pickle and the varying sources of contamination for cover pickle some differences in the types of organisms found in these pickles in different plants are possible.

Correlation of Bacterial Count with Chemical Composition of Pickles

Since bacterial activity is considered responsible for some changes in pickle composition during cure, correlation coefficients were computed between the differences in the logarithms of the number of bacteria present in the cover and spent pickles, or the relative growth rate during cure, and several constituents of the pickle (3). The correlations between the growth rate observed

in 4 and 10% salt media incubated at 20° C. and the mean value of certain components of the pickles appear in Table IX. None of these correlations was statistically significant for the 15 degrees of freedom available. Nevertheless the coefficient between pH and the relative growth rate was high enough to suggest some relation, the growth rate increasing as the pH increases within the range of observed values (4). Computation of this correlation on the basis of the results from individual pickles did not yield significant values, although twice as many degrees of freedom were available. Correlations between the arithmetic increase in bacterial numbers during cure and the nitrate and nitrite contents and the pH of the pickles were also insignificant.

TABLE IX

CORRELATION BETWEEN BACTERIAL GROWTH RATE AND MEAN VALUE OF CHEMICAL CONSTITUENTS OF COVER AND SPENT PICKLES

Quantities correlated			Correlation coefficient (*r*)
Log difference of counts on spent and cover pickles (4% salt agar)	*and*	Salt	−.01
		Nitrate	−.12
		Nitrite	.02
		pH	.45
		Protein	−.33
Log difference of counts on spent and cover pickles (10% salt agar)	*and*	Salt	.19
		Nitrate	.22
		Nitrite	.11
		pH	.36
		Protein	−.01

15 degrees of freedom, r of 0.48 required for 5% level of significance.

Actually a correlation with salt content could hardly be expected. The salt content of pickle varies from about 22 to 28%, and for halophilic organisms variations in salt content at concentrations about 20% have little effect. On the other hand, organisms affected by salt concentration are usually inhibited by concentrations much below this. A correlation between the increase in the number of bacteria, or of nitrate reducing organisms, and the total nitrate reduced might be expected, but under plant conditions it is very difficult to differentiate between the nitrate reduced and that absorbed by the sides since the combined errors applicable to the several determinations are large compared with the quantity of nitrate reduced. Likewise a measure of the total nitrite formed is difficult since the amount that reacts with the muscle proteins is unknown. Until these total quantities can be determined correlations of chemical constituents and bacterial numbers of pickle seem unlikely.

Acknowledgments

In addition to the managements of the various plants and those acknowledged in the first paper of this series, the author is indebted to Drs. J. W. Hopkins and W. H. Cook for advice in making and interpreting the statistical analyses, and to Mr. A. Morrison for assistance in making the counts.

References

1. COCHRAN, W. G. Empire J. Exptl. Agr. 6 : 157-175. 1938.
2. COOK, W. H., GIBBONS, N. E., WINKLER, C. A., and WHITE, W. H. Can. J. Research, D, 18 : 123-134. 1940.
3. COOK, W. H. and WHITE, W. H. Can. J. Research, D, 18 : 135-148. 1940.
4. COOK, W. H. and CHADDERTON, A. E. Can. J. Research, D, 18 : 149-158. 1940.
5. LOCHHEAD, A. G. Progress report of the Dominion Agricultural Bacteriologist for the years 1934, 1935, and 1936. Dominion of Canada, Dept. of Agr. 1938.
6. SNEDECOR, G. W. Statistical methods. Collegiate Press, Inc. Ames, Iowa. 1937.
7. YATES, F. and COCHRAN, W. G. J. Agr. Sci. 28 : 556-580. 1938.

CANADIAN WILTSHIRE BACON

VI. QUANTITATIVE BACTERIOLOGICAL STUDIES ON PRODUCT[1]

By N. E. Gibbons[2]

Abstract

Surface counts of bacteria on the ribs of bacon showed that nutrient agar containing 4% salt incubated at 20° C. gave the maximum number. In sampling the surface of a side of bacon, it was found that the removal of the surface layer of tissue yielded more accurate values than methods based on the removal of organisms by swabs or filter paper impressions.

On the average, the bacterial load on the anterior ribs was greater than on the posterior ribs. Although there were significant differences in the number of bacteria on sides from the same plant, the greatest variation was between sides from different plants.

A visible growth of bacteria or "slime" becomes evident on the average when the logarithm of the number of organisms per sq. cm. exceeds 7.2. Nevertheless, certain sides may appear slimy at log 6.7 per sq. cm., while others will not show this condition at log 8.0 per sq. cm. This variation in the number of organisms present at the visible slime level may result from differences in the flora, different types of growth of the same organism, or variability in the method of detection.

The number of bacteria on the side was found to be correlated with the age of the sides from cure or from packing. The growth rate is slow during the first 8 to 10 days from packing, after which it increases. Sides having an initial load of 100,000 organisms per sq. cm. at packing may be expected to remain free from slime for 20 to 25 days, if stored at 1.1° C. No correlation was obtained between the number of bacteria in the curing pickle and the number on the product.

Introduction

Wiltshire sides are matured for a week to ten days after removal from the curing tank. Whether bacteria play a part in this process is not known, but there is a development of organisms on the surface of the meat which eventually may lead to undesirable features, such as slime or taint.

This study was undertaken to determine the variation in the bacterial load on sides of factory-cured Canadian Wiltshire bacon at the time of export, and the effect of transport or storage on the increase in bacterial numbers and the appearance of slime. For this purpose two sides from each of 22 plants were examined. The treatment of these sides, both before and after reaching the laboratory, has been described elsewhere (2).

Cultural Methods

Some preliminary work was necessary to determine the best medium for growth and the best method of enumerating the bacteria on the surface of bacon. Many of the types found in pickle must be present on the sides when

[1] *Manuscript received February 9, 1940.*

Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. Issued as Paper No. 39 of the Canadian Committee on Storage and Transport of Food. N.R.C. No. 906.

[2] *Bacteriologist, Food Storage and Transport Investigations.*

removed from cure. While conditions may not be favourable for the development of all types present, some are able to grow as shown by the production of slime.

It was found that bacon infusion agars yielded slightly higher counts than beef extract agars, but the difficulty in preparing a clear medium, and the fact that the infusions contained varying amounts of salt, precluded their use as a routine medium. Beef extract, peptone agars were therefore used throughout the investigation.

To determine the optimum salt concentration, samples taken from cured bacon were diluted in brine containing from 0 to 10% salt, cultured on beef extract agar containing the same concentration of salt, and incubated at 20° C. The highest counts were obtained in the range 3 to 6% salt, the numbers falling off rather sharply at higher and lower concentrations. Within this range the maximum usually occurred on 4 to 5% salt. Since there is some advantage in using the lowest salt concentration compatible with good growth, a concentration of 4% salt was chosen with dilution in brine of the same concentration. It is of interest to note that the salt concentration of the bacon is usually about 4 to 5% (3). Consequently, it is evident that the conditions on bacon favour the development of organisms of intermediate salt tolerance.

Occasionally, counts equal to, or even higher than, those on 4% salt were obtained on 10% salt agar. It is conceivable that organisms from pickle, which prefer or are capable of growing at higher concentrations, sometimes persist for a time on the product.

Sampling

Three methods of taking the samples for surface bacterial counts were studied. The first of these, described by Haines (7), consists of outlining a definite area with sterile borers, and then removing a thin layer of the surface meat, or, in the case of ribs, the pleural membrane. Stainless steel borers, cutting a 3 sq. cm. circle, were used in this work. The second method, described by Garrard and Lochhead (4), consists of pressing a piece of filter paper of known area against the surface of the meat for 20 to 30 sec. The third was a swab method, with a piece of sheet aluminium having a 1 × 2 cm. opening used as a mask. In practice the mask was placed against the meat and the exposed area swabbed vigorously with two pledgets of absorbent cotton. The pieces of excised tissue, filter paper, or cotton obtained were in each instance transferred to dilution blanks containing coarse silica sand and shaken vigorously 100 times.

These methods were compared by taking duplicate samples by each method from the 3rd, 5th, 7th, 9th, and 11th ribs of a side of bacon. The position on the rib, from which each of the individual samples was taken, was determined by preparing a random sampling chart. The results were subjected to an analysis of variance which appears in Table I. The mean counts by the three methods show that the numbers obtained by the Haines and swab

techniques are in close agreement on the average, while that by the filter paper procedure is significantly lower. The mean square between duplicates shows that the precision decreases in the order: filter paper impression, Haines, and swab techniques. However, on the small number of observations made, only the swab technique can be said to be significantly less precise than the other methods.

TABLE I

ANALYSIS OF VARIANCE OF COUNTS ON BACON BY THREE METHODS

Method	Log mean no./cm.^{2}	Variance between duplicates		Variance between ribs		F
		D.f.	Mean sq.	D.f.	Mean sq.	
Haines	5.41	5	.028	4	.086	3.07
Filter paper	4.98	5	.013	4	.240	18.46**
Swab	5.40	5	.138	4	.041	0.30

** *Exceeds 1% level of significance.*

The filter paper impression method alone showed a significant difference between the numbers of bacteria on different ribs. Since the other two sampling procedures yielded similar results and showed no differences between ribs, it is concluded that the filter paper method, although precise, gives a less accurate estimate of the actual number of bacteria present. Examination of the detailed results showed that on an arithmetical basis the filter paper method gave about 75% of the count obtained by the Haines method on the 3rd rib and only 15% on the 11th rib. Since a similar gradient was not observed by the other methods on this particular side, and as the rear ribs are usually drier than those nearest the shoulder, it appears that the filter paper method may be affected by moisture conditions on the surface.

This effect of moisture was also observed for cut surfaces of meat. Pieces of filter paper of sufficient size were used to permit a sample to be taken afterwards from the same area by the Haines technique. On somewhat dry surfaces only about 10% of the organisms were removed by the filter paper when compared with the total number removed by the combined methods. On moister surfaces, up to 30% of the organisms could be removed by the filter paper.

The criticism may be made that on meat surfaces the Haines method may also include organisms from the sub-surface layers. However, similar results were obtained on rib surfaces where only the pleural membrane is removed. Since this membrane is not disintegrated when shaken with sand, only surface organisms would be removed.

The swab method is the least precise of the three. It should be mentioned that only after considerable experience with it could the high counts reported be attained. The Haines technique was therefore adopted in this study. Although it has the disadvantage of disfiguring the sides, it gives the best estimate of the actual number of organisms present, and in practice is probably the simplest to use.

Routine Procedure

In the examination of the sides from the various plants all counts were made on the pleural membrane, since this surface has the least opportunity of outside contamination, and slime generally appears there first. Areas of 6 sq. cm. were removed from the pleura over the 2nd and 3rd ribs and over the 9th and 10th ribs, and 3 sq. cm. from the 5th rib. The 5th and 6th ribs were then removed without contamination of the pleura and stored at 1.1° C. in an atmosphere of about 95% relative humidity (over saturated zinc sulphate solution) for 15 to 17 days. The sides were rebaled and stored at 1.1° C. for 10 to 12 days as previously described (2).

At the second sampling 6 sq. cm. samples were taken from all ribs. In all cases dilutions were made in 4% brine and cultured on 4% salt agar. Counts were made after 7 days' incubation at 20° C.

Quantitative Results

Statistical methods (8) have been used to interpret some of the data. Since there was a wide variation in the number of bacteria found, the numbers were converted to logarithms both for convenience and valid statistical analysis (1).

The logarithms of the mean, maximum, and minimum number of organisms per sq. cm. are shown in Table II. From the means it may be seen that there was a slight decrease in bacterial numbers on the pleura from the anterior to posterior ribs both before and after storage. At both samplings the counts for the anterior ribs showed the greatest variation. Since the 5th rib was stored for a longer period, the second sampling from it cannot be compared with the others.

TABLE II

BACTERIAL COUNT ON PLEURAL MEMBRANE OF WILTSHIRE SIDES

(Logarithm of number of organisms per sq. cm.)

	Ribs 2 and 3		Ribs 9 and 10		Rib 5	
	Received	Stored 10–12 days	Received	Stored 10–12 days	Received	Stored 15–17 days
Mean	4.54	6.03	4.21	5.18	4.51	6.94
Maximum	6.27	8.55	5.64	7.41	6.56	9.25
Minimum	2.97	3.48	2.90	3.19	3.42	4.21
Stand. deviation	.67	1.19	.55	.93	.60	1.16
Coeff. of variation	14.8	19.8	13.0	18.0	13.3	16.7

The maximum and minimum values, the standard deviation, and the coefficient of variation (Table II) give some indication of the variation that occurs between the different sides and also between ribs. Since the standard deviation decreases with the decrease in mean number present, the coefficient of variation is fairly constant.

Sources of Variability

The variability shown by the standard deviation in Table II may be divided into that arising from three main sources: (i) the error of diluting, plating, and counting; (ii) differences in the bacterial load of sides from the same plant; and (iii) differences in the product from different plants. The variance due to these three sources is shown in Table III. For all three positions on the side the variance due to differences between sides from different plants is significantly greater than that due to differences between sides from the same plant, which in turn is significantly greater than the experimental error. The greatest source of variability is therefore between plants.

Appearance of Slime

A side may be said to be slimy when the bacteria have increased sufficiently to be visible or tactile. Although not of serious practical importance unless very pronounced, slime is considered to be an indication of improper handling at some stage during the history of the product. If the numbers are sufficient to be discerned, it is probable that taint will soon be detected.

Many of the 5th and 6th ribs that had been stored at 95% relative humidity for 15 to 17 days showed slime, and furnished an opportunity of determining the number of organisms necessary for a visible growth. On arranging the values for slimy and non-slimy sides (Fig. 1), it was found that there was a region in which sides having the same count may or may not appear slimy. Pleural membranes having a count of 5,000,000 per sq. cm. (log 6.70) or less were never slimy; with a count of 93,000,000 per sq. cm. (log 7.97) or over, the pleura was always slimy. Between these limits there were nine slimy and nine non-slimy ribs having an average count of 16,500,000 organisms per sq. cm. (log 7.22 $\pm$ 0.093). It is therefore probable that any side having a count of 16 to 17 million organisms per sq. cm. will be slimy. The counts obtained on the sides stored for a shorter time confirmed these results (Fig. 2). Haines (6) has found that slime becomes visible on stored beef at a similar value (log 7.5).

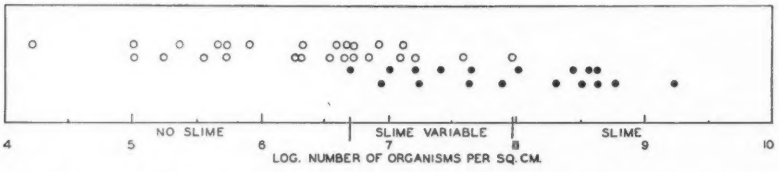


FIG. 1. *Relation between number of organisms on pleural membrane of Wiltshire sides and appearance of slime.*

In this study a visible growth was used as the criterion of slime. In practice a side may also be considered slimy if it feels slippery or if a growth can be detected by running the thumb nail over the surface. These subjective methods of detecting slime are doubtless liable to considerable variability,

TABLE III

ANALYSIS OF VARIANCE OF COUNTS ON BACON FROM 22 PLANTS

Variance	Ribs 2 and 3				Ribs 9 and 10				Rib 5			
	Received		Stored		Received		Stored		Received		Stored	
	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.	D.f.	Mean sq.
Analytical error	86	.0040	82	.0084	87	.0016	85	.0119	86	.0030	86	.0022
Between sides from same plant	22	.4671**	21	1.5617**	22	.2553**	22	1.0472**	22	.3641**	22	1.4774**
Between sides from different plants	21	2.2882**	20	7.0647**	21	1.5696**	21	4.1791**	21	1.8034**	21	6.6714**
Differential change with time between plants	20	2.507**			21	1.816**			21	2.339**		
Average change with time over all plants	1	127.545**			1	63.445**			1	397.096**		

***Exceeds mean square error or intra-plant variance, respectively, 1% level of significance.*

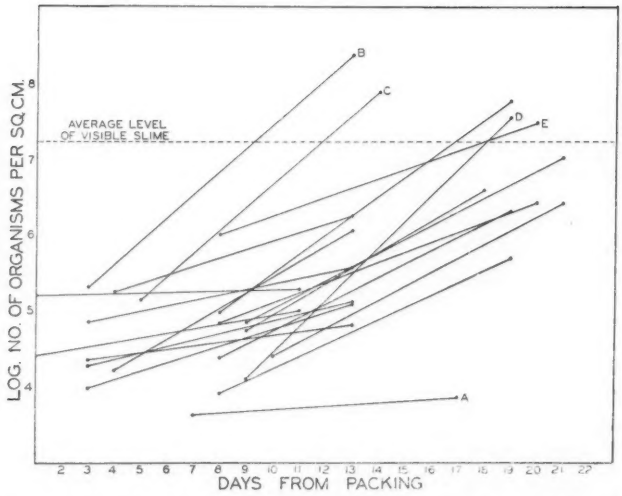


FIG. 2. *Relation between number of organisms on pleural membrane of Wiltshire sides and age from packing.* (*Sides B, C, D, and E showed definite slime.*)

even within a given method. Consequently, variations in the number of organisms present when a side is considered to have become slimy are to be expected. This may account for all or part of the observed variability. However, part of the variation may be real, since the visibility of equal numbers of organisms may be affected by the type of bacteria present, or by the conditions of growth on a particular side.

The significant interaction (differential change with time between plants, Table III) indicated that the bacteria do not increase at the same rate on sides from different plants. This is apparent from the slopes of the lines in Fig. 2 and suggests that the flora of different plants varied. It would appear that the flora of the sides from plant A is quite different from that of sides from plant B. On the other hand, sides from plants B and C, and possibly D, seem to have a similar flora, which grows very rapidly and is soon apparent as slime. Reports on the conditions of sides arriving in England showed that the product from plant B had a higher percentage of slime than that from the others. The conclusion seems justified that, while the majority of plants apparently have very similar types of organisms present on the sides, in a few instances quite different types may be present.

Relation of Number of Bacteria Present to Time

A significant correlation coefficient indicated that there was a relation between the number of bacteria on the sides at successive samplings and the time from the end of cure to each sampling (Table IV). The bacterial count was also correlated with the number of days from the beginning of cure and from packing.

On plotting the data (Fig. 2), this relation was confirmed and another demonstrated. Since the beginning and end of each line in Fig. 2 represent the number of bacteria present at the first and second samplings respectively, the slope of the line indicates the growth rate during this time. It can be seen that, if plants such as A, B, and C are excluded, the growth rate on sides sampled for the first time 2 to 4 days from packing is much less than on sides first sampled 8 to 10 days from packing. Although the lines are based on only two determinations, it is probably safe to conclude that under the conditions of storage there is a period of about 10 to 12 days during which the bacteria develop very slowly on the sides. After this time, development is much more rapid.

Since the appearance of slime depends on the number of bacteria present, it is evident that all sides will eventually become slimy. Apart from those having "abnormal" floras, the original load is important in determining how long the side remains free of slime. Sides having a high initial load (plant E) become slimy before those having a low initial load. From the evidence presented it may be said that, in general, if a side has a count of 100,000 per sq. cm. (about log 5) or less when packed, it will remain free of slime for 20 to 25 days under the usual conditions of transport.

TABLE IV

CORRELATION BETWEEN NUMBER OF BACTERIA ON PRODUCT, NUMBER IN PICKLE, AND TIME

Quantities correlated			Correlation coefficients (r)		
			Ribs 2 and 3	Rib 5	Ribs 9 and 10
Log of no. of organisms per sq. cm. on each side regardless of origin (total 40 sides)	*and*	Days to each sampling from end of cure	.60**	.80**	.56**
		Days to each sampling from packing	.58**	.80**	.57**
Log mean no. of organisms per sq. cm. on 2 sides originating from each of 20 plants	*and*	Days to each sampling from end of cure	.64**		
		Days to each sampling from packing	.61**		
		Days start of cure to each sampling	.63**		
Log mean no. of organisms per sq. cm. on 2 sides from each of 20 plants (1st sampling)	*and*	No. of bacteria per ml. in curing pickle at end of cure (4% salt agar)	.32†		
		(10% salt agar)	.25†		
		(Nut. agar 20° C.)	.28†		
		(Nut. agar 37° C.)	.14†		

** *Exceeds 1% level of significance, 38 degrees of freedom, r of .42 necessary for 1% level of significance for 35 D.f.*

† *15 degrees of freedom, r of .48 necessary for 5% level of significance.*

Relation of Number of Bacteria on Product to Number in Curing Pickle

The organisms found on bacon may come from a number of sources. Garrard and Lochhead (4) point out that the contamination on the sides prior to cure probably plays an important role. It is also possible that the organisms in curing pickle may play a part and the variation found in the number of bacteria in curing pickles (5) might be reflected in the number found on the product. To test this assumption the correlation coefficients shown in Table IV were computed. The number of bacteria on the bacon was not significantly correlated with the number of bacteria in the pickle in which it had been cured. However, the bacon examined varied in its age from cure and had been subjected to the various wiping and packing practices, and shipping conditions followed in the different plants. These factors, among others, are probably more important than the number of organisms in the pickle in determining the number of bacteria on the cured bacon.

Acknowledgment

The author is indebted to Mr. A. Morrison for technical assistance throughout this study.

References

1. COCHRAN, W. G. Empire J. Exptl. Agr. 6 : 157-175. 1938.
2. COOK, W. H., GIBBONS, N. E., WINKLER, C. A., and WHITE, W. H. Can. J. Research, D, 18 : 123-134. 1940.
3. COOK, W. H. and WHITE, W. H. Can. J. Research, D, 18 : 135-148. 1940.
4. GARRARD, E. H. and LOCHHEAD, A. G. Can. J. Research, D, 17 : 45-58. 1939.
5. GIBBONS, N. E. Can. J. Research, D, 18 : 191-201. 1940.
6. HAINES, R. B. J. Hyg. 33 : 175-182. 1933.
7. HAINES, R. B. Department of Scientific and Industrial Research, Food Investigation Board. Special Report No. 45. H.M. Stationery Office, London, England. 1937.
8. SNEDECOR, G. W. Statistical methods. Collegiate Press, Inc., Ames, Iowa. 1937.

CANADIAN JOURNAL OF RESEARCH

Notes on the Preparation of Copy

General:—Manuscripts should be typewritten, double spaced, and the original and one copy submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. All pages should be numbered

Abstract:—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.

Illustrations:—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only. Paper ruled in green, yellow, or red should not be used. The principal co-ordinate lines should be ruled in India ink and all lines should be of sufficient thickness to reproduce well. Lettering and numerals should be of such size that they will not be less than one millimetre in height when reproduced in a cut three inches wide. If means for neat lettering are not available, lettering should be indicated in pencil only. All experimental points should be carefully drawn with instruments.

Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. Small photographs should be mounted on cardboard and those to be reproduced in groups should be so arranged and mounted. The author's name, title of paper, and figure number should be written on the back of each illustration. Captions should not be written on the illustrations, but typed on a separate page of the manuscript.

Tables:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum.

References should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper. The form of literature citation should be that used in this Journal and titles of papers should not be given. All citations should be checked with the original articles.

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.